DEPARTMENT OF HEALTH & HUMAN SERVICES
FOOD AND DRUG ADMINISTRATION

Public Health Service

Memorandum

Date . OCT - 8 1999

9421 '99 OCT 13 P1:51

From Senior Regulatory Scientist, Regulatory Branch, Division of Programs & Enforcement Policy (DPEP), Office of Special Nutritionals, HFS-456

Subject 75-day Premarket Notification for New Dietary Ingredient

To Dockets Management Branch, HFA-305

New Dietary Ingredient:	Siraita Groxvenori (Lo Han Kuo)
Firm:	Nature's Marvel International
Date Received by FDA:	October 6, 1999
90-day Date:	December 24, 1999

In accordance with the requirements of section 413(a)(2) of the Federal Food, Drug, and Cosmetic Act, the attached 75-day premarket notification for the aforementioned new dietary ingredient should be placed on public display in docket number 95S-0316 after ~~December 24, 1999~~ 1/3/2000 jb

Robert J. Moore, Ph.D.

95S-0316

RPT 57

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington, DC 20204

OCT - 8 1999

Nature's Marvel International
U.S. Division
1681 Alta La Jolla Drive
La Jolla, California 92037

Dear Sir:

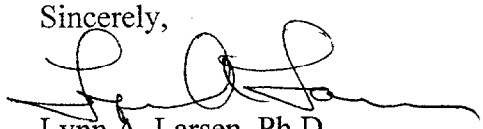
This is in response to your letter to the Food and Drug Administration (FDA) dated September 24, 1999, making a submission pursuant to 21 U.S.C. 350b(a)(2) (section 413 of the Federal Food, Drug, and Cosmetic Act (the Act)) and 21 CFR 190.6. In your letter, you notified FDA of your intent to market Lo Han Kuo Fruit Extract (*Siraitia grosvenorii* (swingle) C. Jeffrey), a substance that you assert is a new dietary ingredient.

The Act, as amended by the Dietary Supplement Health & Education Act of 1994, defines the term "dietary supplement" to exclude products represented for use as conventional foods. 21 U.S.C. 321(ff)(2)(B). In your submission, you state that the ingredient Lo Han Kuo is a new, natural food sweetener. You state in your submission that this ingredient is intended to be used as a sweetener in foods such as low calorie diet and drink supplements, it is a safe alternative to other sweeteners such as saccharine, duicin, and sodium cyclamate, and it can be used in place of sugar as a sweetener. Given the representations made for this product, as cited above, the product is not a dietary ingredient within the meaning of 21 U.S.C. 321(ff) and, therefore, cannot be a "new dietary ingredient" under 21 U.S.C. 350b. Because Lo Han Kuo is not a new dietary ingredient, it is not subject to the notification requirements in 21 CFR 190.6.

Instead, Lo Han Kuo is a conventional food ingredient. Under the Federal Food, Drug, and Cosmetic Act, any ingredient intentionally added to a conventional food must be used in accordance with a food additive regulation unless it is generally recognized as safe (GRAS) among qualified experts for its intended use in food. A food ingredient that is not GRAS or an approved food additive causes a food to be adulterated under 21 U.S.C. 342(a)(2)(C) and cannot be legally marketed in the U.S. If you intend to market Lo Han Kuo as an ingredient in food, it must be an approved food additive or it must be GRAS. Any questions regarding the marketing of this substance in conventional foods should be directed FDA's Office of Premarket Approval (HFS-200), 200 C St., SW, Washington, DC 20204.

Please contact us if we may be of further assistance.

Sincerely,



Lynn A. Larsen, Ph.D.
Director
Division of Programs and Enforcement Policy
Office of Special Nutritionals
Center for Food Safety
and Applied Nutrition

NATURE'S MARVEL

September 24,1999

Office of Special Nutritionals
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C St. SW
Washington, DC. 20204

NEW DIATARY INGREDIENT PREMARKET NOTIFICATION

NAME OF DISTRIBUTOR. NATURE'S MARVEL INTERNATIONAL
ADDRESS. 1681 ALTA LA JOLLA DRIVE. LA JOLLA, CA. 92037
NAME OF THE DIETARY SUPPLEMENT. LO HAN KUO FRUIT EXTRACT. MOMORDICA GROSVENOR
SIRAITA GROSVENORI. LO HAN KUO GLUCOSIDE.
DESCRIPTION, CONDITIONS OF USE, LEVEL OF USE, PLEASE SEE ATTACHED
RECOMMENDED USE LEVEL IN ALL FOODS. 0.25%
SUGGESTED LABELING. EXTRACT OF THE FRUIT OF PARADISE, LO HAN KUO FRUIT EXTRAC

67540

NATURE'S MARVEL INTERNATIONAL
U.S. DIVISION
OFFICE OF STEVE BANYAN
1681 Alta La Jolla Drive • La Jolla, California 92037 • Phone 619.456.4501 Fax 619.459.2428

1. Brief Introduction of the Extract and the Major Technical Requirements

Lo-HAN-KUO [Siraitia grosvenorii (swingle) C. Jeffrey] is a calabash plant, whose ripe fruits are used for health purposes and as a dietary supplement. It is one of the traditional export items from China. After 1949, it's production has made a priority, especially during the 7th - 5 year planing period of the Chinese Government, funding was increased, more manpower and equipment were provided for producing the LO-HAN-KUO. Due to production increases, selling the extra fruit became a problem. This led to a desire to develop more applications where the LO-HAN-KUO can be used. Due to this urgent need to develop more uses for LO-HAN-KUO, and the research funding provided by the China Bank of Agriculture and Investment, led to a series of achievements. It is summarized as follows:

1) For the first time the chemical compositions of the fresh fruit of the LO-HAN-KUO have been determined. There are 5 calabash triterpene glucosides. From the structural studies. it was found that 3 of them are very sweet, and one of them is found the first time that it is the natural sweetner glucoside. NMR techniques were used for the structure determination. We also first time discovered the flavone compositions in the LO-HAN-KUO. We isolated and determined the structures of the grosvenorine from the new flavone glucoside. Those results are very useful for discovering the other applications.
2) New techniques for the extraction process have been developed. A resin extraction method was used which included deposing, regenerating the resins and product refinery. Large scale tests were done many times and then scaled up to a pilot plant with a 1.5 ton production scale. It has been proven that the technique has a short cycle, there is no need for special equipment, it is easy to operate, high production yields and batch to batch reproducibility is very high. The resin is easy to regenerate, produces less waste and generates no hazardous waste. This technique has been

approved for use in part of the production. The herbal extract manufactured from LO-HAN-KUO by using this process has 70% of the total glucosides content and the sweetness is 210 to 250 times sweeter than sugar, depending upon application. The recovery for fresh fruit is 1 % and 3 % by weight for the dried fruit.

3) First time regulated the quality assurance procedures, and the standard operating procedures and those are in effect.

2. Prospect of the Product and the Benefit

This study was based on using the fresh fruits. LO-HAN-KUO is specially grown in China. It grows around many southern Chinese provinces, and has a high yield and many suppliers. Due to the increasing growth of the plant, there is a large supply of the raw material.

Compare LO-HAN-KUO herbal extract with the synthetic sweetner, such as saccharine, duicin and sodium cyclamate, the biggest advantage of using LO-HAN-KUO is that LO-HAN-KUO is safe. is not harmful or toxic to the body. Regular sugar causes tooth decay. People gain weight from the calorie and human immune system is decreased. It can increase the fat content in the blood and cause blood vessel narrowing. For those synthetic sweetners, the toxicity has been tested and some of them have been limited or banned in some of the countries. LO-HAN-KUO has a high sweetness. It is about 210 - 250 times sweeter that sucrose so the caloric content of LO-HAN-KUO is minimal. It is very suitable for low calorie diet and drink supplements, for diabetes patients, people who are overweight and those people who have arteriosclerosis diseases. Meanwhile, LO-HAN-KUO is very soluble in water and ethanol and it is very thermostable. It does not decompose under continuous heating at 100°C for 5 hour and at 120 °C for 12 hour. This property made the process of beverage manufacturing and disinfective process as much easier. LO-HAN-KUO is non-fermentable and it is resistant to molding. It can be stored for long period of time, and has no strange smell, tastes good and smells good.

Since the fresh LO-HAN-KUO is quickly processed, it reduces the loss caused from moldy fresh fruits from storage. It requires less storage space and also cuts down the storage maintenance, disinfection and transportation. This reduces the cost. The by - product from the new technique can also be used for some other purposes, this becomes very economical.

3. Reviews from the Appraisal Committee

The extract of LO-HAN-KUO is an extract from the natural plant specially produced in China. The ripe fruits are used as food and in Chinese medicine. It has been an export item for China for a long time. Since the collaboration between GuangXi YongFu LO-HAN-KUO Manufacturer and the Department of Plant and Development from the Chinese Academy of Medical Sciences, they have discovered a new sweetner only available from China. LO-HAN-KUO's glucoside is 210 times sweeter than sucrose. A new natural sweetner is generated for both domestic markets and the international market, it will benefit the Children, elderly people, and people who need to reduce their sugar intake (such as diabetes patients, overweight people, high blood pressure patients, people having heart disease). LO-HAN-KUO glucoside has high sweetness, low caloric content, only use a small of amount. It tastes good and it is a substitute for sugar used for foodstuff, beverages, medicines, and other industries. It has a great future.

We have reviewed the production process and the quality assurance standards. By using the process which uses the resin to extract the glucoside from LO-HAN-KUO fresh fruits has generated better quality products. It also generated high yields from a number of large scale trials, and 3 production batches from the pilot plant. This process requires fewer production steps, less investment, and produces consistently high quality product from batch to batch. It is also easy to operate and produce no environmental pollutions. Scientifically and economically, it makes more sense.

We also reviewed the initial discovery of the chemical composition analysis from the fresh LO-HAN-KUO fruit. Five calabash triterpene glucoside and one flavone were found from the extraction of fresh fruit, a new natural glucoside was also found - a new LO-HAN-KUO type flavone. New technologies were used for determination the chemical structure.

It is a very difficult research. The organic and inorganic structures attach to the glucoside are also analyzed. This provided scientific evidence for the development and the utilization of this product.

This research is correct and reliable. It targeted the right thing. It has a full data to support the results. It is a new natural sweetner that can be used in the food industry. The new production process is advanced. The new discovery of the new natural sweetner and the utilization of the by -products will benefited the domestic and international markets. It also increases the economic value of the LO-HAN-KUO. It improves the economic development for the grown area and improve the living standards for the farms. It will create a new utilization of the natural resources.

Based on those considerations, we highly recommended to award this research.

Approved by Wang Zhenggang and Chen Xiaoshu

1/11/1993

4. Review Results form the Organizing Committee

Approved by the committee.

Sealed by

the Chinese Academy of Medical Sciences

6/4/1993

5. Review Results from the Appraisal Committee

Approved by the committee

Sealed by the Chinese Academy of Sciences

6/4/1993

6. Documentation Provider and the Name of the Company

A Corpus of Research and Development of LO-HAN-KUO Extract

GuangXi YongFu LO-HAN-KUO Manufacturing Standards
Q/452325 LGZ X50 02-92

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1. Scope and Contents

This procedure has set a number of standards for the manufacturing requirements, testing methods, QC and QA procedures, packaging, storage and transportation.

This procedure is suitable for fresh LO-HAN-KUO and dry LO-HAN-KUO as the raw materials.

2. Manufacturing Standards

GB 601 Manufacturing method for Chemical Reagents and Standard Solutions.

GB2760 Hygiene Standards for Food Additives

GB4789.2 Microbiological Testing for Food and Hygiene, Total Microbe Determination

GB4789.3 Microbiological Testing for Food and Hygiene, Salmonella Determination

GB4789.4 Microbiological Testing for Food and Hygiene. Pathogenic Bacteria Determination

GB5009.3-85 Water Content Determination in the foodstuff.

GB5009.4-85 Dust Determination in the foodstuff.

GB5009.11 Total Arsenic Determination in the foodstuff.

GB5009.12 Total Lead Determination in the foodstuff.

GB7718 Labeling Standards for Foodstuff

ZBX51003 Testing Standards, Regulations, Packaging, Transportation, Storage Standards for Fragrant Fruit Type Solid Drinks.

GuangXi YongFu LO-HAN-KUO Manufacturer Approved on 9/2/1992 and Effective on 9/2/1992

3. Technical Requirements

3.1 Apparent

It should be a light yellow or yellow power, has special fragrance, very sweet and very soluable in water and ethyl alcohol.

3.2 Physical Properties

Table 1

Items	Specifications
Content %	> 70
Sweetness	>210
Absorption $E^{3\%}_{1cm}$ 410 nm	<0.4
Dust %	<0.9
Water Content %	<9
Pb mg/kg	<1.0
As mg/kg	<0.5

3.3 Identification

Take exactly of 10 mg of the sample, dilute it in 1 ml of MeOH, make sure it is dissolved. make it as a sample solution. Take 10 mg of LO-HAN-KUO standard and make a standard solution as the one you made for the sample solution.

Using TLC test to make sure the sample TLC spots match the standard TLC results (Attachment 2: 1990, volume 1 Appendix, Page 57 TLC method).

3.4 Microbe Standard

Table 2

Items	Specifications
Total Microbe #/gram	<1000
Salmonella #/gram	Undetectable
Bacteria #/gram	Undetectable
Live mites, eggs #/gram	Undetectable

4. Testing Procedures

4.1 Test by Appearance and Taste:

Take 10 g of the sample, use eye to check it's color. It should be light yellow or yellow, it should have a nice fragrance. Take 5 g of it, dissolve it in water. The resulting solution should be tasted very sweet.

4.2 Physical Test:

4.2.1 Content determination

Generate a standard curve:

Weigh exactly 30 mg of LO-HAN-KUO reference standard and add to a 5 ml volumetric flask, add 70% ethanol in water and dilute to mark and shake. Take exactly 10, 20, 30, 40, 50, ul to 10 ml test tubes which can be capped. Use hot air to evaporate out the solvent (Not too high temperature) add 0.2 ml of freshly made 5% vanillin - glacial acetic acid solution and add 0.8 ml of perchloric acid, heat it in 60 ^{0}C water bath for 15 minutes, take it out and cool it with cold water immediately. Add 5 ml of glacial acetic acid, shake. Use the solvent as the blank, to generate a concentration vs. absorbance curve at 590 nm using spectroscopy (the spectroscopic method is described in Appendix 1: Volume 1, page 51, 1990 Chinese Pharmacopoeia). All the test should be done with one hour.

Concentration Determination:

Weigh exactly 30 mg of sample, dissolve it in 5 ml volumetric flask with 70% ethanol, diluted to mark, shake it well. Dilute 40 ul of the solution to a 10 ml test tube with cap and determine the absorbance. The concentration of the sample from the standard curve and calculate the concentration as follows:

Content X = c /(8 * W) x 100%

c: Concentration obtained from the standard curve (Unit, μg)

W: Sample weight (unit: mg)

4.2.2 Sweetness Test:

Compare the 2% sucrose solution with 210 fold dilution of the 2% of the LO-HAN-KUO solution.

4.2.3 Absorption Determination:

Weigh sample and make up a 2 mg/ml solution and determine the absorbance (Spectroscopic method is in Appendix 1: Volume 1, page 51, 1990 Chinese Phamacopoeir) at 410 nm.

4.2.4 Dust Determination:

Under the guidelines of GB 5009.4 -85

4.2.5 Water Content Determination:

Under the guidelines of GB 5009.3 -85

4.2.6 Lead Content Determination:

Under the guidelines of GB 5009.12

4.2.7 Arsenic Content Determination

Under the guidelines of GB 5009.11

4.3 Identification:

Weigh 10 mg of the sample, add 1 ml of MeOH to make sure it dissolves. Use it as sample solution. Weigh 10 mg of the LO-HAN-KUO reference standard and make up the solution in the same way.

Run TLC tests (Attachment 2: 1990, volume 1 Appendix, Page 57 TLC method). Spot both reference standard and sample, develop in $CHCl_3$ - MeOH - H_2O (40: 23: 5). Spray with 10% phosphomolybdic acid in ethyl alcohol, heat at 110°C for 5 minutes. The spots shown on the TLC plate for the sample should match Rf the spots of the reference standard.

4.4.1 Total Microbe Determination

Under the guidelines of GB4789.2

4.4.2 Salmonella Determination:

Under the guidelines of GB4789.3

4.4.2 Pathogenic Bacteria Determination

Under the guidelines of GB4789.4

5. Quality Assurance Regulations:

5.1 Each cycle of the production has one batch number.

5.2 Samples are random tested with each batch and once it passes all the requirements, it will be issued a pass certificate before the product can go out of the factory.

5.3 Under the normal production conditions, bacteria level, dust content, water content, absorption, and sugar content are mandatory tested routinely on every batch. Other tests are done randomly at a regular basis.

5.4 If is test is failed, a second test can be done. If the second test still failed, then those batches will be failed.

5.5 During the guarantee period, if there is concern from the customer about the quality. The problem can be discussed or brought to an arbitrator. If the quality problem is due to the inappropriate transportation, or storage, the manufacturer will not be responsible for the loss. The transportation, storage firm should be responsible.

6. Label, Packaging, Transportation and Storage

6.1 Label

The label should have the product name, manufacturer name, address, registered trade mark, production date (or batch #), expiration date, product standard code and net weight.

6.2 Packaging

All the packing material should meet the requirements of " The People's Republic of China Food and Hygiene Regulations (trial version)", Under the guideline of GB 10790.

6.3 Quality Guaranteed.

Name List of the Major Contributions

#	Name	Age	Education	Major	Title	Company	Major Contributions
1	Chen Dihua	51	BS or above	Organic Chemistry	Scientist	Plant Dept. of the Chinese Academy of Sciences	Project leader for chemical analysis and quality assurance and production design
2	Chang Qi	30	BS or above	Pharmacy	Associate Scientist	Plant Dept. of the Chinese Academy of Sciences	Chemical analysis, technical requirements and quality research
3	Jiang Chunfa	29	BS or above	Chemical Engineering	Engineering	Plant Dept. of the Chinese Academy of Sciences	Technical requirements
4	Si Jianyong	30	BS or above	Pharmacy	Associate Scientist	Plant Dept. of the Chinese Academy of Sciences	Chemical analysis and production research
5	Liu Xibin	58	BS or above	Mechanics	Senior Scientist	Plant Dept. of the Chinese Academy of Sciences	Coordinator and process testing
6	Shen Liangang	22	Vocational School		Technician	Plant Dept. of the Chinese Academy of Sciences	Process research
7	Huong Bin	26	BS or above	Chinese Medicine	Associate Scientist	Plant Dept. of the Chinese Academy of Sciences	Process testing

Name List of the Appraisal Committee

Title in the Committee	Name	Company	Major	Title	Signature
Chairman	Wang Zhenggang	Medical Research Institute of the Chinese Academy of Sciences	Pharmacy	Scientist	Signed
Vice Chairman	Chen Xiaoshu	Nutrition and Foodstuff Research Institute of China Academy of Prevention Medicinal Science	Nutrition	Scientist	Signed
Member	Shen Guohua	Beijing Foodstuff Research Institute	Foodstuff	Senior Scientist	Signed
Member	Zhang Yuzhong	Chinese Medicine Research Institute	Analytical Chemistry	Scientist	Signed
Member	Sha Shiyan	Medicinal Research Institute of the Chinese Academy of Medical Sciences	Analytical Chemistry	Scientist	Signed
Member	Sun Weillian	Plant Research Institute of the Chines Academy of Medical Sciences	Plant Chemistry	Scientist	Signed
Member	Bi Zhi	Sino-Japanese Hospital Clinical Research Institute	Plant Chemistry	Scientist	Signed

The Certificate of the Glucoside of the LO-HAN-KUO as a Dietary Supplement

Glucoside of the LO-HAN-KUO is an extract from the fresh fruit LO-HAN-KUO. It is a dietary supplement. It is co-developed by the Plant Resources Research Institute of the Chinese Academy of Sciences and GuangXi YongFu Manufacturer. It is a dietary supplement and only available in China.

LO-HAN-KUO has been used as a fruit, summer beverages and it has been used as a Chinese healthful herb by the people of GuangXi and GuangDong Provinces. It was recorded in 1997 in the China Pharmacopoeia as a Chinese herbal extract. In 1987, LO-HAN-KUO was listed as the first group of items that can be used as a dietary supplement by the Administration of Chinese Medicine of the Ministry of Hygiene. It showed that LO-HAN-KUO is safe for human consumption. The glucoside is the main content in the LO-HAN-KUO, and is useful as a dietary supplement.

GuangXi YongFu Manufacturer

6/3/1997

Attachments:

LO-HAN-KUO glucoside production diagram

2. The Modern Research of LO-HAN-KUO

2.1 In 1975, it was reported by Lee, et al[1] that there was a triterpene contained in LO-HAN-KUO, but there was no structure. In 1983, Takekimatsuhar from the University of Dedao in Japan discovered that LO-HAN-KUO contains much fructose. They also found that 3 kind of sweet LO-HAN-KUO glucosides. All those discoveries were done with the commercial available LO-HAN-KUO sold in Hong Kong. Later he isolated and structurally determined 7 components from the LO-HAN-KUO purchased in Macao. In 1992, Chen Dihua from the Department of Plant Resources and Development of the Chinese Academy of Medicinal Sciences did further research on the chemical composition. He was able to isolate 6 kinds of glucoside. There were also results on isolated glucoside and its contents of low molecular weight sugar, hydrolysis amino acids, some of the vitamins, fatty acid and inorganic compounds. Before Chen's research, Xu Weiqun from GuangXi Plant Research Institute also did the measurement of the amino acids content. Zhao Jifu et al did the sweetness testing: Chen Hongbing (from the Plant Resources and Development of the Chinese Academy of Sciences) did the toxicity studies on the LO-HAN-KUO extract.

2.2 Takekeharu [2] determined there are mogrosides IV, formula $C_{54}H_{92}O_24 \cdot H_2O$, mogroside V, formula $C_{60}H_{102}O_29 \cdot H_2O$ and mogroside VI, formula $C_{66}H_{112}O_{34}$ from the dry LO-HAN-KUO. They also determined the glucoside on V - is mogrol and its glucoside structure [3]. it is approved as triterpene.

2.3 Matsuki [4] separated and determined 7 components. Including the structures mentioned above, mogrosides IV, and mogroside V, there was also siamenoside I, formula $C_{54}H_{92}O_{24} \cdot 7/2\ H_2O$,11 - DXO - mogroside V, formula $C_{60}H_{100}O_{29} \cdot 7/2\ H_2O$, mogroside II E, formula $C_{42}H_{82}O_{19}$ and a very small amount of mogroside III, formula $C_{48}H_{82}O_{19}$ (compare with the mogroside III E, they are sterioisomers, they have different optical properties). Siamenoside I was determined as the sweetest triterpene, the sweetness is 563 times sweeter than 5 % of regular sugar when the concentration is diluted to 1/1,000,000.

2.4 Chen Dihua, et al[6] did further research with the fresh LO-HAN-KUO. They obtained the total glucoside and then isolated the mogroside II E, mogroside III, mogroside IV, mogroside V and the new discovery of the neomogroside, formula $C_{66}H_{112}O_{34}$. Among them, mogroside is the main component of the LO-HAN-KUO, which takes up to 0.5 % of the total weight of the fresh LO-HAN-KUO. They also found two kinds of flavones, the structure is -3-O-α–L- rhamnose- 7 - O- β - D-Glucosido-(1-2)- α -L- Rhamnose and phenyl -3, 7- α -L- dirrhamnoside. The first one is the natural product. Later on they determined the remaining product other than the extract. They were able to determine the low molecular weight sugar, hydrolyzed amino acids, some of the vitamins, fatty acids, and some of the inorganic component. The oil extracted from the solid waste takes about 0.8 % of the total fresh weight. It contains unsaturated fatty acid (49.9 %), saturated fatty acid (7.7 %), palmic acid (7.2 %) and stearic acid (4.4 %). Vitamin A in the oil is 8 IU/g. Other than the extracts, there are glucose which takes up 0.8 % of the total weight of the fresh fruit, fructose which takes up to 1.5 % of the total fresh weight, vitamin B1, B2 which take up to 3.38 % and 1.23 % (mg/g). The hydrolysis of various amino acids and the content is listed in Table 1.

Table 1 - Hydrolysis of the Various Amino Acid and their Content:

	Contents g/100 g		
Amino Acids	Fresh LO-HAN-KUO after removal water	water after removed the sugar content	solid waste
aspartic acid	0.9	1.56	0.61
threonine	0.25	0.28	0.25
serine	0.35	0.39	0.37
glutamic acid	0.55	0.97	0.87
glycine	0.36	0.32	0.44
alanine	0.53	0.82	0.40
cystine	0.24	0.23	0.16
valine	0.47	0.48	0.48
methionine	0.2	0.15	0.14
isoleucine	0.4	0.38	0.38
leucine	0.5	0.41	0.54
tyrosine	0.28	0.34	0.29
phenylalanine	0.32	0.31	0.41
lysine	0.31	0.22	0.37
histine	0.18	0.20	0.14
arginine	1.28	1.08	0.68
proline	0.27	0.19	0.27
total	7.38	8.36	6.87

The inorganic contents are listed in Table 2.

Table 2 - Inorganic Contents

	Content ppm	
Elements	Fresh LO-HAN-GUO after removal the water	water after removed the sugar content
Al	16.7	18.63
B	12.9	51.79
Ba	15.4	13.88
Ca	2221	11049
Cr	2.25	4.59
Cu	7.70	14.77
Fe	159	97.36
K	16089	44314
Mg	1138	6971
Mn	20.82	30.2
Cu	1.26	4.4
Na	86.5	4280
Pb	6.08	13.84
Zn	11.4	265
S	1314	5889

2.5 In 1986, Xu Shenchun [7] did many chemical composition studies, he reported that the protein is 7.1 - 7.8 % of the total dried fruit. He also reported the contents of the 18 out of the 19 amino acids from the hydrolysis for four different LO-HAN-GUO (different grower). In 1980, he measured 339 - 487 mg of Vitamin C per 100 g of fresh fruit. He also measured 0.1864 ppm of selenium (Se) which is 2 - 4 times more than the grain [8].

Selenium was reported that it has anti-heart disease, anti aging and anticancer effects.

2.6 In 1992, Zhao Jifu et al from the Tanjing Engineering Institute did the LO-HAN-KUO extract sweetness testing. The test was used sucrose as the threshold value (0.5 %, weight percentage). Using the international threshold stimulant testing method, it was reported that the extract from LO-HAN-KUO is 210 times sweeter than sucrose (t test, 5 % standard deviation) [9]. It was reported that the extract did not decompose when heated at 120 °C for 12 hours.

2.7 There has also been progress on the pharmacology and toxicity studies. In 1983, Takekimatsuharu [10] used mice, rabbits and dogs for tests. The LO-HAN-KUO extract resists the intestines shrinking cause by BaCl or acetyl chlorine, he makes the intestines move easily. At a high dosage, it reduced the blood pressure. In his toxicity study reports, it stated LD 50 testing (Half fatality number) on mice that LO-HAN-KUO extract (freeze dry) is greater than 10 g/kg; Chen Hongbin [11] used mice for LD 50 testing, he used the maximum concentration (60 %) and maximum volume (0.4 ml/ 10g wt) poured the solution (equivalent to 24 g/ kg body weight) down the throat, and observed the activity of the animal and found no fatalities. The animals were under observation for two weeks after the dosage and there was no abnormal behavior and no fatalities observed. Based on the regulations of the Ministry of the Hygiene which stated that if there is no fatality during the first stage of the rapid toxicity testing with a dosage of 10 g/ kg of the body weight, it is not necessary to do the LD 50 testing (Half fatality testing).

3. LO-HAN-KUO Extract's Future

In the southern part of the China, drinking LO-HAN-KUO tea is as a status of the person for more than hundred of years. Due to it's good taste, people like it. Recent years, LO-HAN-KUO has been used in herbal remedies. There are more than 20 years of history of people using dry LO-HAN-KUO as a tea. Since the 80's, LO-HAN-KUO has always been a product requested by the foreign trade department. It has been exported to most countries in Southeast Asia.

References

[1] L. H. Lee, Experientia, 1975; 31: 533.

[2] Takekimatsuharu, 3, Journal of Pharmacology, 1983; 103: 1151.

[3] ibid 1983, 103 : 1151; 1983; 103: 1167.

[4] K. Matsumoto et al, 1990; Chem. Pharm. Bull. 38 (7): 2030.

[5] R. Sasai et al, 1989; Agric. Biol. Chem, 53 (12): 3347.

[6] Chen Dihua et al; A Corpus of the Research, Development and the Utilization of Chinese Medicine LO-HAN-KUO; 1993, Beijing.

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The Chemical Composition Analysis of Fresh LO-HAN-KUO (I) The Isolation of Triterpenoid Glycosides and Their Structural Determination

Abstract: Chinese fruit LO-HAN-KUO [Siraitia grosvenori (swingle) C. Jeffrey] is specially cultivated in China and it is used as an herb. In this article, 6 glucoside compounds were extracted from the fresh fruit LO-HAN-KUO: Neomogroside **5**, and the known cucurbitane glucoside-mogroside II E, **1**, mogroside III, **2**, mogroside IV, **3**, mogroside V, **4** and a non-cucurbitane glucoside **7**. **3 - 5** are very sweet. **4** is the major component of the LO-HAN-KUO and has about 0.5% of the fruit weight. **5** is a new sweet glucoside, there is only trace amount in LO-HAN-KUO. The structures of **1 - 5** were determined by using their spectral data (^{1}H and ^{13}NMR, ^{13}C-^{1}H COSY, ^{1}H-^{1}H COSY and NOE Difference Spectroscopy) plus chemical reaction methods. The non-cucurbitane triglycoside is not sweet, its structural determination is in progress.

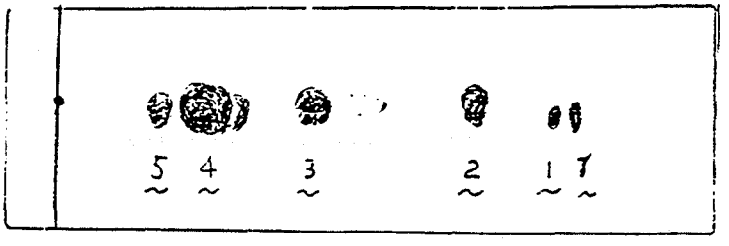
Keywords: LO-HAN-KUO genus, cucurbitane triglycoside, LO-HAN-KUO, herbal extract.

LO-HAN-KUO is classified as the calabash family [Siraitia grosvenori (swingle) C. Jeffrey] and is special vine plant cultivated in China. It is cultivated in YongFu and other counties of the northern part of GuangXi autonomous region. The ripe fruit is used as Chinese herb and tastes sweet. It is beneficial to the respiratory and digestive systems. The fruit is used as a dietary supplement and made into a summer drink by the people in GuangXi and GuangDong provinces. The healthful efficacy of LO-HAN-KUO is recorded in all editions of Chinese pharmacopoeia after 1977[1]. Japanese scientists Takemoto[2] and Matsumoto[3] had studied the chemical compositions of the dry fruit from markets in Hong Kong and Macao. They found 6 glycosides and one aglycone, all

of them are cucurbitane glycosides. According to literature[4], those compounds are the effective components of LO-HAN-KUO. During the course of study the utility of LO-HAN-KUO, we have found that there are many advantages of extracting the glycosides from the fresh fruit over the dried one. From the same quantity of the fresh fruit, the product extracted from the fresh fruit has better yield and lighter color than that from the dried fruit. The operation process is easier for fresh fruit than for the dried one. In addition, due to the lower price of the fresh fruit, it costs less to make the sweet glycosides, most of the production process is starting from the fresh fruit. It has not been reported yet whether the major sweetener composition in the fresh fruit is the same as that in dry fruit. Further study has to be done on this issue. Therefore we have carried out isolation process from the enriched glycoside mixture and we have found 5 cucurbitane glycosides and one non-cucurbitane glycoside. Upon analyzing their spectroscopy data and carrying out chemical tests, we have determined the structures of 5 components. They are mogroside II E (**1**), mogroside III (**2**), mogroside IV (**3**), mogroside V (**4**), and meomeogogroside (**5**). All of them are glucoside of mogrol (**6**) and they are diglucoside, triglucoside, tetraglucoside, pentaglucoside and hexaglucoside of **6** respectively. Components **3**, **4** and **5** are very sweet and they contribute to the sweetness of the LO-HAN-KUO. Component **4** is the major content of the LO-HAN-KUO and it takes up to 0.5% of the fresh fruit weight. Due to components **3**, **4** and **5** contain more sugar groups and they are all the same glycoside, it is very difficult to separate them as well as to determine their structure. The assignment of the NMR spectra is very difficult due to the heavily overlap of the signals on their ^{1}H or ^{13}C NMR spectra. It is very hard to determine the connecting position between sugar and aglycone as well as and between sugar groups. Even though components 3 and 4 are known compounds, all literatures [2, 3, 5] did not give assignments of their NMR spectra for the sugar part. In our study, we not only took the 1D NMR spectra (^{1}H and ^{13}C) and 2D NMR spectra (^{13}C-^{1}H COSY and ^{1}H-^{1}H COSY), but also applied the NOE difference spectroscopy. Some signal overlaps of

the sugar part have been effectively separated and assigned. This result is never achieved in the study on the glycoside on the LO-HAN-KUO before. We have compared the ^{1}H and ^{13}C data of **1 - 5** with those of **6**, the ^{1}H NMR chemical shifts of 4, 5 are listed in Tables 1, 2, 3 and the ^{13}C shifts of **1 - 5** are listed in Table 4 and 5. The non-cucurbitane glycoside **7** is the trace component in LO-HAN-KUO and not sweet, its structure assignment is in progress.

TLC analysis of the glycoside components from the fresh LO-HAN-KUO

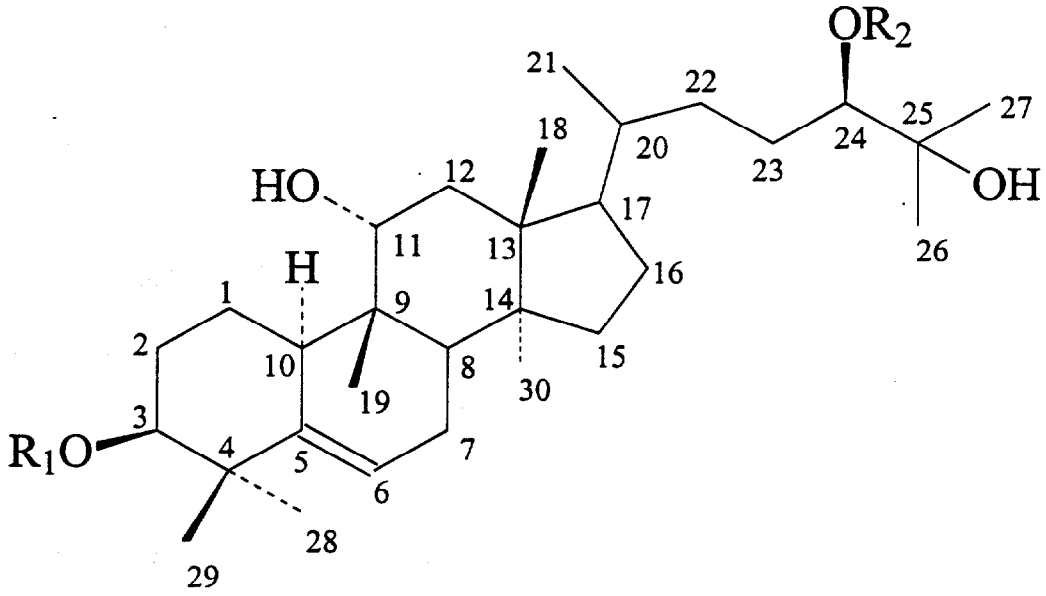


Silica Gel plate

$CHCL_3$-MeOH-H_2O (40:23:5, homogeneous phase)

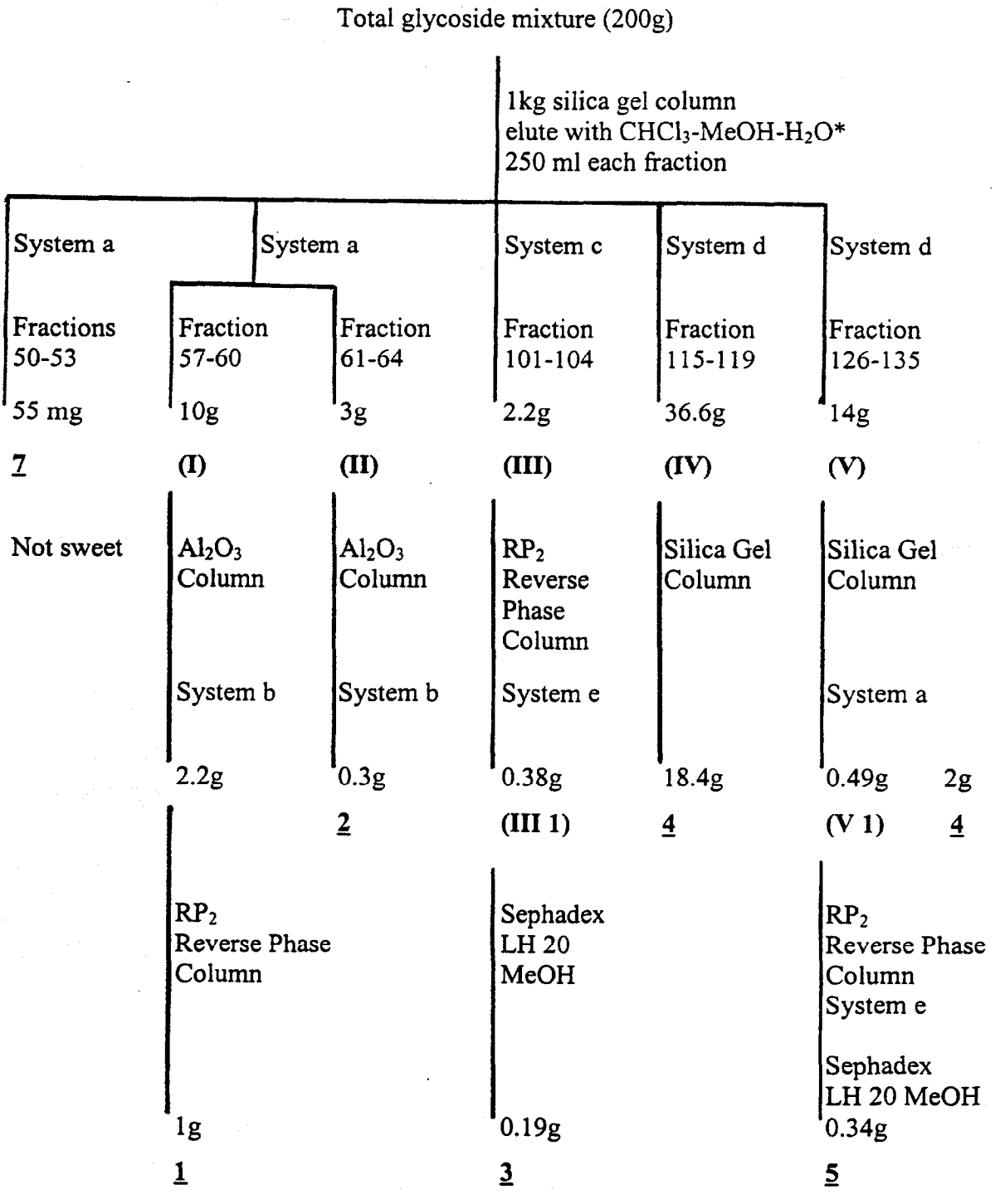
Spray with 10% phosphomolybdic acid in ethyl alcohol and heat to stain

Relative ratio 1:2:3:4 = 2.7: 12.2: 18.4: 66.7 (TLC scan results)



	R1	R2
1	Glc	Glc
2	Glc	Glc—(6-1)—Glc
3	Glc (I)—(6-1)—Glc (II)	Glc (III)—(2-1)—Glc (IV)
4	Glc (I)—(6-1)—Glc (II)	Glc (IV)—(6-1)—Glc (V); Glc (IV)—(2-1)—Glc (VI)
5	Glc (I)—(6-1)—Glc (II); Glc (II)—(2-1)—Glc (III)	Glc (IV)—(6-1)—Glc (V); Glc (IV)—(2-1)—Glc (VI)
6	H	H

Figure 1 Separation Flow Chart for LO-HAN-KUO Glycosides



*Elute system: a: 6.5: 3.5: 1 d: 5: 5:1
b: 7.5:2.5:1 e: 60% MeOH-H_2O
c: 6:4:1

1, Formula $C_{42}H_{72}O_{14}$, white powder, M/Z = 800 by FDMS; NMR results (Table 4) indicate that **1** has the same skeleton as **6**, in its ^{13}C NMR spectrum, δ 107.4 and δ 106.0 ppm indicate **1** is diglucoside. TLC acid hydrolysis indicates its sugar is glucose. ^{1}H NMR gives terminal protons δ 4.87 (1H, d, J = 7.8 Hz) and δ 4.98 (1H, d, J = 8.0 Hz) ppm, this indicates that the glucosidic bond is in β configuration. Compare the ^{13}C NMR data of **1** and **6**, chemical shifts of C_3 (δ 88.0ppm) and C_{24} (δ 90.8ppm) is obviously down field, this indicates the glucosidic bond is between C_3 and C_{24}. Therefore, we assign **1** as mogroside II E.

2, white powder, m/z = 986 $[M+H+Na]^+$, has a formula of $C_{48}H_{82}O_{19}$, Compare the ^{13}C NMR spectra of **2** with **1** (Table 4), they all have the similar ^{13}C chemical shift for their main frame. This means that **1** and **2** have the same skeleton of mogrol. The only difference is that **2** has three sugar terminal groups, which means **2** is a triglucoside. Result from TLC acid hydrolysis indicates that all three sugars are all glucose. The same as **1**, δ 88.0ppm in ^{13}C NMR spectra of **2** indicates that C_3 is connected to a glucose. For the remaining two sugar, δ 92.6 ppm indicates that the glucosidic bond is located at C_{24} position. δ 70.5ppm indicates a 6-1 connection between two sugars, that is, the are gentiobiose. ^{1}H NMR give sugar terminal protons at δ 4.84, 4.92 and 4.98 ppm, coupling constant J = 7.7, 7.4, 7.5 Hz respectively, they are all in β configuration. So, **2** should be mogrolyl-3-O-β-glucopyranose-24-O-β-gentiobios, it is named mogroside III.

3. $C_{54}H_{92}O_{24}$, ^{1}H NMR and ^{13}C NMR have shown that it has 4 sugar groups, this indicates that **3** is a tetraglucoside. Compare ^{13}C NMR data of **3** and **2** (Table 4), **3** and **2** have the same aglycone part, **3** also should be the glucoside of mogrol. TLC acid hydrolysis indicates all sugars in **3** are glucose. The same as **2**, chemical shifts of C_3 (δ 87.4ppm) and C_{24} (δ 92.6ppm) indicate the glucosidic bonds are located at C_3 and C_{24}. Analysis of ^{13}C-^{1}H COSY and NOE difference spectroscopy, the connecting pattern between sugar in **3** is determined. From the ^{13}C NMR spectrum of **3**, δ 62.5, 62.5, 63.2,

70.2 ppm represent the four terminal carbons, δ 70.2 also indicates a 1-6 connection. Chemical shift at δ 82.8 ppm indicates a 1-2 connection[3]. From NOE difference spectroscopy of **3**, irradiation at terminal proton (δ 4.78ppm), give enhancement at 6 positions, the broad single peak at δ 3.64 is C_3H of aglycone, it has a cross section with δ 87.4ppm in ^{13}C-^{1}H COSY. Therefore, the sugar with terminal proton at δ 4.78 ppm should be located inside and is connected at C_3 position. In addition, signals at δ 4.14 (I_{6b}), 4.64 (I_{6a}) are also enhanced, and δ 4.14 (I_{6b}), 4.64 (I_{6a}) have cross section with δ 70.0 ppm (-CH_2O-), this indicate they are the two protons of C_6 of sugar I and sugar I and sugar II have 1-6 connection. Irradiation at δ 5.05 ppm (II $_1$) also results signal enhancement at δ 4.14 and 4.64 ppm. This gives additional evidence for a 1-6 connection between I and II. Upon irradiation the terminal proton at δ 4.80 ppm (III $_1$) signal enhancement at δ 3.65 (C_{24}-H) is observed, δ 3.65 (C_{24}-H) has a cross section with δ 91.7ppm (C_{24}) in ^{13}C-^{1}H COSY, it indicates that sugar III is connected at C_{24}. Signal at δ 4.10ppm is also enhanced and peak at 4.10ppm has cross section with δ 82.8ppm, so sugar IV and sugar III has 1-2 connection. Therefore, **3** is a compound formed by C_3 of mogrol connected to gentiobiose, and C_{24} of mogrol connected to sophorose, it is mogroside IV.

4. White power, m/z = 1310 $[M+H+Na]^+$ with FDMS, ^{13}C and ^{1}H NMR indicates it has five sugar groups, so **4** is a pentaglucoside. Acid hydrolysis has approved all sugar groups are glucose. Comparison ^{13}C NMR data of **4** with **3** indicates that **4** is also glucoside of mogrol. Therefore **4** has formula of $C_{60}H_{102}O_{28}$. The aglycone part of **4** gives similar or equivalent chemical shifts to that of mogrol (except C_3 and C_{24}), this indicates that the glucosidic bonds are formed at C_3 and C_{24} positions. To elucidate the structure of **4**, its glucosidic bond configuration and the connection pattern between sugars have to be determined. From the coupling constant of the five terminal protons (J = 7.7-8.0 Hz), all glucosidic bonds have β configuration. The connection pattern

between sugars are determined by analysis ^{13}C-^{1}H COSY, ^{1}H-^{1}H COSY, and NOE difference spectroscopy. Chemiscal shifts of aglycone part are listed in Table 4 by analysis all spectra of **4** and comparison the ^{13}C data of mogrol. All remaining signals are belong to sugar parts of **4**, the chemical shifts of five terminal carbons is assigned as δ 62.8, 63.0, 63.7, 70.2, 70.4 ppm. δ 70.2 and 70.4 ppm indicate that there are two 1-6 connections (chemical shifts of $-CH_2O-$) and δ 82.7 ppm indicates there is a 2-1 connection.

From the NOE difference spectroscopy of **4**, signal enhancement is observed at 6 locations upon irradiation at δ 4.77 results. The broad single peak at δ 3.67 is C_3-H of aglycone, it has a cross section with δ 87.4 (C_3) in ^{13}C-^{1}H COSY. The sugar with terminal proton at δ 4.47 is the inner sugar connected to C_3 position of aglycone. Other enhancements are located at δ 3.87 (I_2), 4.13 (I_3), 4.03 (I_5), 4.28 (I_{6b}), 4.72 (I_{6a}). The last two have cross section with δ 70.4 ($-CH_2O-$) in ^{13}C-^{1}H COSY, this indicates they are protons at 6 position of sugar I, sugar I and Sugar II have 6-1 connection. Enhancement at δ 4.00 (II_2), 3.93 (II_3), 4.24 (II_5), 4.28 (II_{6b}), 4.72 (I_{6a}) is observed by irradiation at δ 5.12 (II_1) (refer to Figure), enhancement at 4.28 (II_{6b}), 4.72 (I_{6a}) further proves the 6-1 connection of sugar I and II.

In the ^{1}H-^{1}H COSY spectrum, δ 3.78 is correlated to δ 4.77, the proton at δ 3,78 is belong to I_2. The carbon at δ 75.4 is correlated to δ 3.78 in ^{13}C-^{1}H COSY, it is belong to sugar I_2. The assignment of I_3, I_5 and their protons is based on the enhancement (δ 4.13, 4.03) by NOE and the corresponding carbons (δ 78.4, 77.3) is assigned according to the cross over in ^{13}C-^{1}H COSY in combination the fact of carbon chemical shifts of glucose (chemical shift of C_3 is downfield relative to that of C_5[3]). It is determined that carbon I_3 at δ 78.4, carbon I_5 at δ 77.3 ppm, the proton I_3 at δ 4.13, and the proton I_3 at δ 4.03. The proton at C_4 position of glucose is far apart from the terminal proton[4], there is no NOE enhancement observed. Except C_6, C_4 of glucose appears at the most upfield, which is around 73.0 ppm[3], therefore the carbon at δ 71.7 is assigned as I_4 carbon. Further, the

corresponding to I_4 proton is assigned at δ 3.91 according to ^{13}C-^{1}H COSY. Other ^{13}C and ^{1}H chemical shifts are also assigned by the same means, we are not list all of them out.

Further irradiation at δ 4.88 (IV_1) results in enhancement at δ 3.74 (C_{24}-H) and δ 3.74 is correlated to δ 91.7 (C_{24}) in ^{13}C-^{1}H COSY, this indicates sugar IV is connected to C_{24} of aglycone. In addition, obvious enhancement at δ 3.93 (IV_{6b}), 4.02 (IV_5), 4.13 (IV_2), 4.21 (IV_3), is also observed. Since δ 4.13 is correlated to δ 82.7 (IV_2) in ^{13}C-^{1}H COSY, position 2 of sugar IV is connected to sugar VI and. δ 3.93 is correlated to δ 70.2 (IV_6), position 6 of sugar IV is connected to sugar V. Irradiation at the last terminal proton δ 4.83 (IV_1) in NOE difference spectroscopy results in enhancement at δ 3.93 (IV_{6b}), 4.01 (IV_3), 4.02 (IV_1), and 4.20 (IV_5) (refer to figure). These results further prove that sugar IV and sugar VI have 2-1 connection, sugar IV and sugar V have 6-1 connection.

From the results obtained, the C_3 position of aglycone is connected to diglucoside (I 6-1 II) and the C_{24} position is connected to triglucoside (IV 6-1 V, -IV 2-1 VI). Therefore **4** is determined as mogrolyl-3-O-[β-D-glucopyranosido (6-1)-β-D-glucopyranose]-24-O-{[β-D-glucopyranosido (2-1)][β-D-glucopyranosido (6-1)-β-D-glucopyranose]}, mogroside V in short.

5 is white powder, FBMS give m/z 1471 $[M+Na]^+$, acid hydrolysis gives only glucose, the aglycone part is determined as mogrol, its formula is $C_{66}H_{112}O_{34}$. ^{13}C NMR of **5** gives 6 terminal carbons and this indicates **5** is the hexaglucoside of mogrol. Compare the ^{13}C chemical shifts of **5** and mogrol (**6**), chemical shifts of C_3 and C_{24} of **5** are obviously downfield. This indicates the glucosidic bonds are also formed at C_3 and C_{24} positions. The connection pattern between sugar and aglycone, sugar and sugar is determined through ^{13}C-^{1}H COSY, NOE difference spectroscopy of **5** as well as comparison with those of **4**. ^{1}H NMR indicates coupling constant of the terminal protons of all sugar are

between 7.4~7.8Hz, so all sugars have β configuration. ^{13}C NMR indicates that there are two 6-1 connections (δ 70.2 and 70.5 ppm) and two 2-1 connections (δ 82.5, 82.6 ppm). ^{13}C chemical shifts of aglycone of **5** is assigned in Table 4 according to cross over in ^{13}C-^{1}H COSY and comparison to spectral data of **4**. δ 87.6 and 91.9 ppm are assigned as C_3 and C_{24} respectively and these two carbons are connected to sugar. In NOE difference spectroscopy, irradiation of the proton at δ 4.79 (sugar I $_1$) results in obvious enhancement of signal for proton correlated to C_3 of aglycone. This result indicates this proton is the terminal proton of the inner sugar. In addition, Enhancement is also observed at δ 4.30 (I $_{6a}$), 4.15 (I $_3$), 4.04 (I $_5$), 3.87 (I $_2$), 4.21 (I $_4$). δ 4.30 is correlated to δ 70.5 ppm (-CH_2O) in ^{13}C-^{1}H COSY, this indicates position 6 of sugar I is connected to sugar II. Due to the chemical shift of I $_{6b}$ proton is overlapped with that of terminal proton of sugar I (δ 4.78), I $_{6b}$ proton is also excited. Therefore, signal of I $_4$ is affected and signal enhancement is observed for I $_{6a}$ and I $_5$.

Enhancement is observed at δ 4.18 (II $_2$), 4.22 (II $_3$), 3.85 (II $_5$), 4.02 (II $_6$), 4.30 (I $_{6a}$), and 4.78 (I $_{6b}$) upon irradiation at δ 5.16 (II $_1$). δ 4.18 (II $_2$) is correlated to δ 82.5 ppm (carbon II $_2$) in ^{13}C-^{1}H COSY, 4.30 (I $_{6a}$) and 4.78 (I $_{6b}$) are correlated to δ 70.5 ppm (-CH_2O-). These results indicate connection between sugar II $_2$ and III and further prove the connection between sugar II and I $_6$. δ 4.02 is correlated to δ 61.7 (-CH_2OH) and it indicates II $_6$ is not connected to sugar.

Upon irradiation, signal enhancement is observed at δ 4.04 (III $_2$), 4.22 (III $_3$), 3.89 (III $_3$), 3.83 (III $_5$), 4.32 (III $_6$), it indicates sugar III is connected to sugar II $_2$. δ 4.32 (III $_6$) is correlated to δ 61.7 (-CH_2OH), it indicates III 6 is not connected to sugar.

Irradiation the terminal proton of IV (δ 4.89) results in signal enhancement at δ 3.74 (C_{24} of aglycone), 3.94 (IV $_{6a}$), 4.05 (IV $_3$), 4.21 (IV $_3$), and 4.18 (IV $_2$) ppm. The first enhancement indicates IV is an inner sugar connected to C_{24} of aglycone. 4.18 (IV $_2$) is correlated to δ 82.6 ppm (carbon IV $_2$) in ^{13}C-^{1}H COSY, a connection between IV $_2$ and sugar VI is determined. In addition, 3.94 (IV $_{6a}$) is correlated to δ 70.2 ppm (-CH_2O-) in

^{13}C-^{1}H COSY, indicates connection between sugar IV $_6$ and sugar V. These results are further proved by irradiation the two sugar terminal protons δ 5.48 (IV $_1$) and 4.85 (V $_1$) ppm, obvious enhancement is observed at δ 4.18 (IV $_1$) and 3.91 (IV $_6$) ppm.

From all results obtained, C_3 and C_{24} of **5** are connected to three sugar groups, the sequence of connection is I 6-1 II 2-1 III, IV 6-1 V, IV 2-1 VI. **5** is mogrolyl-3-O-[β-D-glucopyranosido (6-1)-β-D-glucopyranosido (2-1) -β-D-glucopyranose]-24-O-{[β-D-glucopyranosido (6-1)][β-D-glucopyranosido (2-1)-β-D-glucopyranose]. **5** is a new nature sweet glucoside, it is named as neomogroside.

Experimental

IR (KBr) is taken on Perkin-Elmer 983G; NMR's are taken on FX-100 or Bruker AM-500, C_5D_5N as solvent NMR solvent, TMS as intern al standard; FDMS was taken on MAT-90 Mass spectrometer.

Silica is from QingDao Ocean Chemical Plant; AlO_3 is from Shanghai Wusi Chemical Reagent Manufacturer; RP_2 reverse phase silica and RP_{18} reverse phase TLC plates are from Merck; Sephadex LH 20 is from Shanghai Chemical Reagent Manufacturer.

Raw materials and extractives of LO-HAN-KUO used in experiment are provided by YongFu Pharmaceutical Manufacturer form the GuangXi Autonomous Region.

Solvent systems used for TLC are mix solvent of $CHCl_3$-MeOH-H_2O, a: 6.5:3.5:1; b: 7.5:2.5:1; c: 6:4:1 (homogeneous); d: 5:5:1 (homogeneous); e: 60% MeOH-H_2O.

Extraction:

5.5 kg black LO-HAN-KUO paste (mobile, equivalent to 30 kg fresh fruit) form YongFu Pharmaceutical Manufacturer form the GuangXi Autonomous Region is added 3 times

water. The mixture is passed through the pre-treated enriching resin column, 317g light yellow glycoside mixture is obtained.

Apply 200g glycoside mixture to a column with 1kg silica gel, elute with various solvent system according to the flow chart. Components 1-6 are obtained.

Composition analysis

1, $C_{42}H_{72}O_{14}$, white powder, IR ν max (cm^{-1}): 3210 (OH), 1640 (C=C). FDMS: m/z 800, ^1H NMR (100 MHz): δ 4.87 (1H, d, J = 8.7 Hz), 4.98 (1H, d, J = 8.0Hz) (terminal protons). ^{13}C NMR (refer to Table 5, 4)

TLC acid hydrolysis[6]: silica G plate is made with 0.4% CMC-Na and is backed at 105°C for a hour and cooled to room temperature. Sample is dissolved in water and is spotted onto the plate, the plate is placed into the developing chamber containing concentrated HCl at room temperature for 50 minutes. The plate is taken out and placed under IR lamp to remove HCl moisture. Glucose is spotted as standard and the plate is developed with n-BuOH-HAc-H_2O (3:1:1). The plate is sprayed with aniline (0.93g)-benzene dicarboxylic acid (1.66g)-n-BuOH saturated with water (100ml) and baked at 105°C for 10 minutes to stain. Only the brown spot corresponding to glucose is visualized.

2, $C_{46}H_{82}O_{19}$, white powder, IR ν max (cm^{-1}): 3400 (br, OH), 1640 (C=C). FDMS m/z 986 $[M+H+Na]^+$, 824 $[M+H+Na\text{-}Glc]^+$. ^1H NMR (100M Hz): δ 4.84 (1H, d, J = 7.7 Hz), 4.92 (1H, d, J = 7.4 Hz), 4.98 (1H, d, J = 7.5 Hz) (terminal protons), 0.85, 0.92, 0.92, 1.16, 1.31, 1.31, 1.43 and 1.43 ppm (8x3H, d, $8CH_3$). ^{13}C NMR (refer to Table 4, 5).

TLC acid hydrolysis: the same as for **1**, only the brown spot corresponding to glucose is found.

3, $C_{54}H_{92}O_{24}$, white powder, IR ν max (cm^{-1}): 3400 (OH), 1640, 890 (C=C), ^1H NMR (500 MHz): 5.05 (1H, d, J = 7.8 Hz), 4.78 (1H, d, J = 8.0 Hz), 4.74 (1H, d, J = 7.4 Hz), 4.73 (1H, d, J = 7.1 Hz), (terminal protons). ^{13}C NMR data (refer to Table 4, 5).

TLC acid hydrolysis: the same as for **1**, only the brown spot corresponding to glucose is found.

4, $C_{60}H_{102}O_{29}$, white powder, IR ν max (cm^{-1}): 3400 (OH), 1640, 890 (C=C), FDMS m/z 1310$[M+H+Na]^+$, 1286 $[M]^+$, 1147 $[M+Na-Glc]^+$, 1129$[M-Glc-H2O]^+$, 985$[M+Na-2Glc]^+$, 823$[M+Na-3Glc]^+$, ^1H NMR (500 MHz) (refre to Table 1, 2), ^{13}C NMR data (refer to Tbale 4, 5).

Acid Hydrolysis: In a safety bottle, it is added 100mg of **4** and 1.5 ml of 5% H_2SO_4, the bottle is heated at 70°C for 6 hours. The reaction mixture was added 3ml water and extracted with n-BuOH saturated with water. The aqueous phase was neutralized with 10% NaOH and all solvent is removed by heating, TLC analysis indicates that only spot corresponding to glucose is found.

5, $C_{66}H_{112}O_{34}$, white powder, IR ν max (cm^{-1}): 3400 (OH), 1660 (C=C); FABMS m/z 1471 $[M+Na]^+$, ^1H NMR and ^{13}C NMR data (refer to Table 1, 3, 4, 5).

Acid Hydrolysis: the same method for **4**, TLC analysis indicates that only spot corresponding to glucose is found.

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LO-HAN-KUO Chemical Composition Analysis (II)
Flavone Glycoside Composition and Structural Determination

Abstract: Two flavone glycosides were obtained from the fresh Chinese fruit LO-HAN-KUO. From the analysis of their ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY and NOE difference spectroscopy, the structures were determined as Kaempferol-3-O-α-L-Rhamnose-7-O-[β-D-Glucosido-(1-2)-α-L-Rhamnoside] (VI) and Kaempferol-3,7-α-L-dirrhamnoside (VII). VI is a new natural component and named as grosvenorine.

Keywords: LO-HAN-KUO, grosvenorine.

LO-HAN-KUO is classified as the calabash family [Siraitia grosvenori (swingle) C. Jeffrey] and is special vine plant cultivated in China. The major chemical composition of dry LO-HAN-KUO is triglucoside. For comparison the chemical composition of the fresh fruit and that of dried one, we have carried out the study of chemical composition of the fresh LO-HAN-KUO. In addition to isolation of 5 cucurbitane triglucosides[1], two flavone glycosides were first isolated, Kaempferol-3-O-α-L-Rhamnose-7-O-[β-D-Glucosido-(1-2)-α-L-Rhamnoside] (VI) and Kaempferol-3,7-α-L-dirrhamnoside (VII). The study of their structures is reported in this article.

Grosvenorine (VI): light yellow needle crystal, mp 218-220°C. Formula is determined as $C_{33}H_{40}O_{19}$ according to Mass, ^{13}C NMR and ^{1}H NMR analysis. IR analysis indicates OH group (3200 cm^{-1}), C=O group (1860 cm^{-1}). It reacts with HCl-Mg and a deep red color is resulted. Molish reaction is positive. UV λmax in MeOH: 245 (sh), 265, 315 (sh), 345 nm, they are the absorption peaks of flavone type compound, so VI is flovane triglycoside. ^{13}C NMR gives three terminal carbons (δ 105.5, δ 101.8, and 97.3 ppm) and indicates VI is a triglycoside. Paper chromatography after acid hydrolysis of VI indicates the sugars are glucose and rhamnose. The coupling constant of the terminal proton of

glucose is J = 8 Hz in ^{1}H NMR, this indicates glucose adopts a β configuration. The coupling constant of the terminal proton of rhamnose is J = 2 Hz, so rhamnose adopts a α configuration. There are 19 O's in its formula, the sugar parts contain 13 O's atoms and two O's from the skeleton of flavone. No evidence of OCH_3 and other groups containing oxygen from ^{13}C NMR, so the remaining 4 O's should be in OH groups. ^{1}H NMR only gives two free phenol OH groups at δ 12.5 and 10.2 ppm (disappear upon D_2O exchange), therefore another two OH groups are glycosidated. VI is flavone glycoside containing two sugar chains and two free phenol OH groups. Upon addition of NaOMe into the solution, band I of the UV spectrum of VI is shifted to red by 40 nm and its intensity does not change, there should be a OH group at position 4'. Addition of $AlCl_3$/HCl results the red shift 40 nm of band I indicates there is OH group at 5 position. From δ 6.8 (1H, d, J = 2Hz) and 6.5 (1H, d, J = 2Hz) ppm from ^{1}H NMR, there two protons at meta-position on the aromatic ring and there should be a substituent at position 7. δ 7.8 (2H, d, J = 8.5 Hz) and 6.9 (2H, d, J = 8.5 Hz) indicate an A_2B_2 system and there is only substituent on ring B because another sugar chain only can be at position 3. From these results, the aglycone of VI should be 3, 5, 7, 4'-tetrahydroxyl flavone. The connection pattern of the sugar is determined according to the analysis of ^{1}H-^{1}H COSY and NOE difference spectroscopy. Upon irradiation of the terminal proton at δ 5.5 ppm on rhamnose, protons at 6, 8 positions of ring A receive stronger signal enhancement, the inner sugar at position 7 should be rhamnose. Irradiation of the terminal proton at δ 4.40 ppm on glucose also results in signal enhancement of H_6, H_8, this indicate position 7 is connected to a disaccharide. Irradiation of the terminal proton at δ 5.0 ppm on rhamnose results in weak signal enhancement at positions 5' and 6' on ring B. This indicates that there is a rhamnose connected to position 3. The proton at position 2 of rhamnose which correlated to the terminal proton (δ 5.30 ppm) of rhamnose at position 7 is at δ 3.92 ppm. From the ^{13}C-^{1}H cosy, the corresponding carbon at position 2 is at δ 79.7 ppm, it is shifted to downfied by 9.7 ppm. Therefore the glucose is connected at the position 2 of

the rhamnose. Irradiation of the terminal proton of glucose also results in signal enhancement at δ 3.97, this indicates it is a 1-2 connection between glucose and rhamnose. We conclude that VI is Kaempferol-3-α-L-rhamnose-7-O-[β-D-glucosido-(1-2)-α-L-rhamnoside, refer to scheme for its structure.

Mogroside VII: light yellow needle crystal, mp 187-189°C, HCl-Mg reaction give positive and molish reaction is also positive. The UV spectrum in anhydrous MeOH gives bands at 264 and 345 nm, they are the characteristic bands for of flavone type compound. This indicates that VII is a flavone glycoside. Comparison of the ^{13}C NMR data of VII and VI indicates that VI has additional six carbons from glucose and all other carbons have similar chemical shifts for both. Therefore VII is dirhamnose glycoside of kaempferol. Upon acid hydrolysis of VII, the aqueous phase is extracted with ethyl acetate and the resulted product is the aglycone of the glycoside. TLC analysis and mp determination of this aglycone with kaempferol as standard have proved it is kaempferol. TLC analysis of the aqueous phase only give the color spot corresponding to rhamnose standard. By comparison the ^{13}C NMR data of VII and kaempferol, chemical shifts of C_3, C_7 positions are upfield –0.8 and 0.9 ppm, the chemical shifts of C_2, C_6, C_8 are downfield 10.0, 1.2, 1.2 ppm. These results indicate C_3 and C_7 are connected to rhamnose. Therefore VII is kaempferol-3, 7-α-Ldirhamnose.

Experimental

Melting point is taken on a Fisher-Johne apparatus (not calibrated); NMR is taken on Bruker 500, DMSO-d_6 is used as solvent and internal standard; Mass spectroscopy is taken on a MaT-711; IR (KBr) is taken on Perkin-Elmer 983; UV is taken on Philips Phy Unicam PU 800.

Extraction

200g glycoside mixture (supplied by GuangXi Yongfu Pharmaceutical Manufacturer) is applied to a short column with 1kg silica gel. Elute with $CHCl_3$:MeOH:H_2O (7:3:1) and collect every 200 ml as fractions. A light yellow crystal from fractions 88 to 93 is obtained and it is recrystalized in CH_3OH and H_2O (3:1) to yield VI (70mg). Combine fractions 68-72 and apply it to a polyamide column. Elute with 60% MeOH and yield a yellow powder. It was recrystalized in MeOH to yield VII (62mg).

Composition Analysis

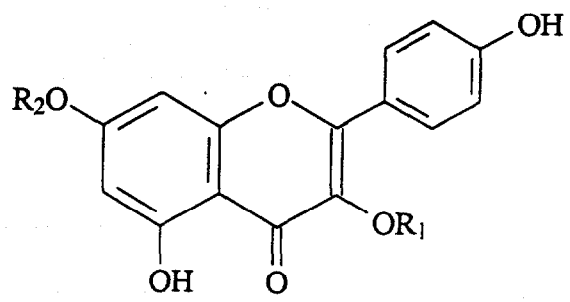
Grosvenorine (VI): mp 218-220°C, HCl-Mg reaction give positive, molish reaction is also positive; IR, ν_{max}^{KBr} (cm^{-1}): 3410, 1660, 1600, 1500, 1210, 1190, 840; UV, λ_{max}^{MeOH} (nm): 265, 345, addition of NaOMe: 245 (sh), 268, 385, addition of $AlCl_3$: 234 (sh), 275, 300 (sh), 342, 395, addition of $AlCl_3$/HCl: 255 (sh), 275, 300 (sh), 342, 395, addition of NaOAc: 265, 370, addition of NaOAc/H_3BO_3: 265, 345; FAB-MS m/z: 763 $(M+Na)^+$, 741 $(M+H)^+$, 595 $(M\text{-}Rha + 1)^+$, 286 $(M\text{-}2Rha\text{-}glu)^+$. EI-MS: m/z (%) 286 (100), 285 (27), 258 (6.4), 229 (5), 213 (3.3), 153 (4), 146 (13); ^1H NMR (DMSO-d_6) δ (ppm): 7.8 (2H, d, J = 8.5 Hz, H2', H6'), 6.9 (2H, d, J = 8.8 Hz, H3', H5'), 6.8 (1H, d, J = 2Hz, H8), 6.5 (1H, d, J = 2Hz, H6), 10.3 (4-OH), 12.6 (5-OH), 3-4 (m, proton form sugar), 5.9 (1H, d, J = 22 Hz, Rha-H1), 5.2 (1H, d, J = 2Hz, Rha'-H1), 4.4 (1H, d, J = 8.0 Hz, Glc-H1), 0.8 (3H, s, Rha-H6), 0.79 (3H, s, Rha'-H6). ^{13}C NMR (DMSO-d_6), δ (ppm): 156.1 (C-2), 134.6 (C-3), 177.9 (C-4), 160.9 (C-5), 99.5 (C-6), 161.4 (C-7), 94.6 (C-8), 157.7 (C-9), 105.8 (C-10), 120.3 (C-1'), 130.6 (C-2', C-6'), 115.6 (C-3', C-5'), 160.1 (C-4'). Glc: 105.2, 73.8, 76.7, 69.3, 76.7, 60.8, 7-Rha: 97.3, 79.7, 70.4, 71.9, 70.6, 17.7. 3-Rha: 101.9, 70.0, 70.8, 71.2, 69.8, 17.3.

Acid hydrolysis: 20 mg of VI was added 5% H_2SO_4/ethanol (1:1), the mixture is refluxed for 4 hours, TLC analysis indicates the completion of hydrolysis. It is extracted with

ethyl acetate three times, the recovered aglycone has the same R_f value as that of kaempferol. Paper chromatography of the aqueous phase only gives Glc and Rha.

VII: light yellow needle crystal, mp 187-189°C, HCl-Mg reaction gives positive and molish reaction is positive. UV, λ_{max}^{MeOH} (nm): 246 (sh), 267, 385, addition of $AlCl_3$: 274, 300 (sh), 345, 395, addition of $AlCl_3$/HCl: 274, 300 (sh), 345, 395, addition of NaOAc: 265, 358, 400 (sh), addition of NaOAc/H_3BO_3: 265, 345. EI-MS m/z (%): 286 (100), 185 (27), 258 (6), 229 (5), 213 (3.3), 153 (4), 146 (13). ^{13}C NMR (DMSO-d_6) δ (ppm): 156.1 (C-2), 134.5 (C-3), 177.9 (C-4), 160.9 (C-5), 99.5 (C-6), 161.7 (C-7), 94.6 (C-8), 157.8 (C-9), 105.8 (C-10), 120.4 (C-1'), 130.8 (C-2', C-6'), 115.4 (C-3', C-5'), 160.1 (C-4'), 3-Rha: 101.8, 70.2, 70.7, 71.8, 70.1, 17.5, 7-Rha: 98.4, 70.0, 70.7, 71.6, 69.8, 17.9.

Acid hydrolysis: hydrolysis as normal method, extracted with ethyl acetate four times, the recovered aglycone has the same R_f value as that of kaempferol. Paper chromatography of the aqueous phase only gives Rha.



	R1	R2
grosvenorine	Rha	Rha$\overset{2\text{-}1}{\text{——}}$Glc
mogroside VII	Rha	Rha

Table 1 ^{1}H NMR chemical shifts (ppm) of aglycone of **4**, **5** in C_5D_5N

Proton	**4** (J, Hz)	**5** (J, Hz)
1-H	2.96 (1H, d, 10.4)	2.96 (1H, br. s)
	2.00 (1H, dd, 10.5, 12.0)	2.00 (1H, dd, 10.5, 12.0)
2-H	2.43 (1H, dd, 10.5, 3.0)	2.44 (1H, br. s)
	2.10 (1H, m)	2.10 (1H,m)
3-H	3.67 (1H, br. s)	3.67 (1H, br. s)
6-H	5.43 (1H, d, 6.4)	5.45 (1H, d, 6.0)
7-H	2.28 (1H, dd, 7.2, 6.4)	2.25 (1H, m)
	1.65 (1H, d, 7.3, -)	1.65 (1H, m)
8-H	1.61 (1H, d, 7.3)	1.59 (1H, d, 7.3)
10-H	2.78 (1H, d, 12.1)	2.77 (1H, d, 12.0)
11-H	4.17 (1H, dd, 5.0, 11.5)	4.18 (1H, dd, 5.0, 11.5)
12-H	2.15 (2H, m)	2.15 (2H, m)
15-H	1.15 (2H, m)	1.15 (2H, m)
16-H	1.49 (1H, m)	1.49 (1H, m)
	2.15 (1H, m)	2.15 (1H, m)
17-H	1.87 (1H, m)	1.89 (1H, m)
18-H	0.91 (3H, s)	0.91 (3H, s)
19-H	1.09 (3H, s)	1.10 (3H, s)
20-H	1.52 (1H, m)	1.52 (1H, m)
21-H	1.07 (3H, d, 7.7)	1.10 (3H, d, 7.7)
22-H	1.78 (2H, m)	1.78 (2H, m)
23-H	2.43 (2H, m)	2.45 (2H, m)
24-H	3.74 (1H, d, 9.0)	3.73 (1H, d, 9.1)
26-H	1.44 (3H, s)	1.44 (3H, s)
27-H	1.33 (3H, s)	1.31 (3H, s)
28-H	0.92 (3H. s)	0.90 (3H, s)
29-H	1.09 (3H, s)	1.10 (3H, s)
30-H	1.49 (3H, s)	1.50 (3H, s)

Table 2 ^{1}H NMR chemical shifts (ppm) of the sugar of **4** in C_5D_5N

	Sugar				
Proton	I (J, Hz)	II (J, Hz)	IV (J, Hz)	VI (J, Hz)	V (J, Hz)
1	4.77 (J, 7.8)	5.12 (d, 8.0)	4.88 (d, 7.8)	5.42 (d, 7.8)	4.83 (d, 7.7)
2	3.87 (dd, 8.0, 8.5)	4.00 (dd, 8.0, 8.5)	4.13 (dd, 8.5, 8.0)	4.06 (dd, 8.0, 9.3)	4.02 (m, 8.0, 8.5)
3	4.13 (dd, 8.5, 8.5)	3.93 (dd, 8.5, 11.3)	4.21 (dd, 9.0, 8.5)	4.17 (dd, 9.3, 8.0)	4.01 (m, 8.5, 8.0)
4	3.91 (dd, 8.5, 9.3)	4.02 (m, 8.0, 8.5)	4.21 (dd, 8.5, 9.0)	4.08 (dd, 8.5, 9.3)	3.91 (dd, 9.3, 8.5)
5	4.03 (m, 4.7, 8.0, 8.5)	4.23 (m, 8.5, 9.0)	4.02 (m, 7.4, 8.0, 8.5)	3.91 (m, 3.5, 8.5, 9.3)	4.20 (m, 8.5, 9.0)
6a	4.28 (dd, 11.2, 4.8)	4.28 (dd, 11.2, 4.8)	4.86 (dd, 7.8, 11.3)	4.48 (dd, 12.4, 10.4)	4.45 (d, 12.4)
6b	4.72 (d, 11.2)	4.42 (d, 12.4)	3.93 (dd, 11.3, 8.5)	4.30 (dd, 3.5, 11.2)	4.30 (dd, 3.5, 11.2)

Table 3 ^{1}H NMR chemical shifts (ppm) of the sugar of **5** in C_5D_5N

	Sugar					
Proton	I (J, Hz)	II (J, Hz)	III (J, Hz)	IV (J, Hz)	V (J, Hz)	VI (J, Hz)
1	4.79 (d, 7.8)	5.16 (d, 7.8)	5.05 (d, 7.7)	4.89 (d, 7.8)	4.85 (d, 7.6)	5.48 (d, 7.7)
2	3.87 (dd, 7.7, 9.5)	4.18 (dd, 8.0, 10.5)	4.04 (br, s)	4.18 (dd, 8.0, 10.5)	4.02 (br, s)	4.08 (dd, 8.0, 9.5)
3	4.15 (dd, 9.0, 10.5)	4.22 (d, 8.5)	4.22 (dd, 8.5, 10.0)	4.21 (dd, 8.5, 10.5)	4.22 (dd, 8.5, 10.5)	4.19 (dd, 9.0, 10.5)
4	4.21 (dd, 9.0, 10.0)	4.10 (dd, 9.0, 10.0)	4.10 (dd, 9.0, 10.0)	4.21 (dd, 9.0, 10.0)	3.92 (dd, 10.5, 7.0)	4.10 (dd, 9.0, 10.0)
5	4.04 (m)	3.85 (dd, 9.0, 7.0)	3.88 (dd, 10.0, 7.5)	4.05 (dd, 9.0, 8.4)	3.85 (dd, 9.0, 7.0)	3.92 (9.0, 7.0)
6a	4.30 (dd, 9.0, 7.8)	4.02 (m)	4.28 (m)	3.94 (8.0, 7.5)	4.28 (m)	4.30 (m)
6a	4.78 (dd, 9.0, 7.7)		4.50 (t, 12.0, 13.0)	4.90 (9.0)	4.50 (t, 12.0, 13.0)	4.50 (t, 12.0, 13.0)

Table 4: ^{13}C NMR chemical shifts of aglycone of **1**, **2**, **3**, **4**, **5**, and **6** (ppm, 125 MHz, C_5D_5N)

Glucoside

Carbon	1	2	3	4	5	6
1	26.5	26.7	26.4	26.6	26.9	25.0
2	29.8	29.8	29.5	29.4	29.5	30.0
3	88.0	88.0	87.4	87.4	87.4	76.2
4	42.6	42.8	42.4	42.3	42.4	42.2
5	144.1	144.6	144.3	144.5	144.5	144.3
6	118.5	118.9	118.4	118.2	118.5	119.1
7	24.8	24.9	24.7	24.7	24.9	24.5
8	43.6	44.1	44.7	43.7	43.7	43.6
9	40.3	40.6	40.2	40.3	40.3	40.2
10	37.0	36.7	36.9	36.8	36.6	36.9
11	77.9	78.1	78.2	78.0	78.0	77.8
12	41.3	41.3	40.9	41.2	41.2	41.2
13	47.5	47.8	47.5	47.6	47.6	47.4
14	49.8	50.2	49.8	49.8	49.8	49.8
15	34.8	35.1	34.7	34.7	34.7	34.5
16	28.5	28.1	28.0	28.6	28.6	28.4
17	51.1	51.5	51.1	51.5	51.2	51.0
18	17.3	17.6	17.3	17.2	17.1	17.3
19	27.2	27.3	26.7	27.1	27.2	26.7
20	36.7	36.7	36.5	36.5	36.5	36.3
21	19.1	19.4	19.3	19.2	19.1	18.9
22	33.7	33.3	33.4	33.4	33.4	34.2
23	29.8	29.8	29.7	29.6	29.5	29.0
24	90.8	92.6	92.6	91.7	91.6	79.0
25	72.2	73.1	72.8	72.8	72.9	72.7
26	25.5	24.7	24.4	24.7	24.7	25.8
27	26.6	26.7	26.4	26.3	27.8	26.3
28	19.6	19.9	19.6	19.5	19.5	19.3
29	28.0	27.3	27.8	27.8	26.4	27.3
30	26.5	26.7	26.4	26.3	26.4	26.2

Table 5: ^{13}C NMR chemical shifts of the sugar part of 1, 2, 3, 4, and 5 (ppm, 125 MHz, C_5D_5N)

Glucoside

Carbon		1	2	3	4	5
C_3-O-Glc	1'	107.4	107.1	106.6	106.8	106.7
(Inner)	2'	75.4	75.4	75.2	75.4	75.3
(I)	3'	78.7	78.3	78.3	78.4	78.4
	4'	71.8	71.6	71.4	71.5	71.7
	5'	78.2	78.1	78.2	77.3	77.9
	6'	63.1	63.1	63.2	70.4	70.5
C_3-sugar	1'			105.1	105.3	105.5
(Terminal,	2'			75.2	76.0	76.0
6-1	3'			76.4	78.1	78.3
(II)	4'			71.4	71.9	71.8
	5'			78.3	78.6	78.3
	6'			62.5	63.0	62.9
C_3-sugar	1'					105.4
(Terminal,	2'					75.1
2-1)	3'					76.7
(III)	4'					71.6
	5'					78.4
	6'					62.8
C_{24}-O-Glc	1'	106.0	104.7	104.8	103.5	105.6
(Inner)	2'	75.4	75.6	82.9	82.7	82.6
(IV)	3'	78.2	78.1	78.2	78.7	78.6
	4'	71.8	71.9	72.9	72.8	72.9
	5'	78.2	76.6	77.2	77.3	77.2
	6'	62.8	70.5	70.2	70.2	70.2
C_{24}-sugar	1'		106.3	106.1	106.4	106.3
(Terminal,	2'		75.4	79.2	78.2	77.9
6-1	3'		78.5	78.3	78.4	78.4
(V)	4'		71.9	72.6	72.1	72.6
	5'		78.5	78.2	78.7	78.7
	6'		62.9	62.5	63.2	63.7
C_{24}-sugar	1'				104.9	104.8
(Terminal,	2'				75.3	75.2
2-1)	3'				78.5	78.5
(VI)	4'				71.8	73.2
	5'				78.4	77.9
	6'				62.8	61.7

Package B:
Regulatory Issues of the Lo-Han-Kuo Project

Document Number: NLA-Bf3-011999
Submitted to Nature's Marvel International by Sinotech
January 31, 1999

Project Scope and Background

Nature's Marvel International (NMI) intends to submit a self-affirmed GRAS (Generally Regard as Safe) petition to FDA. Preliminary information containing the following 5 Chinese documents (referred as Package A) were provided by the manufacturer of Lo-Han-Kuo extract in China:

- AC-1: Result Certificate -- Research & Development of Lo-Han-Kuo Product (by Institute of Chinese Medicine, 1/11/93)
- AC-2: Manufacturing Standard Operating Procedures (by Guangxi Yongfu Lo-Han-Kuo Factory, 9/2/92)
- AC-3: Certificate of Lo-Han-Kuo Glucosides as a Food Additive (by Guangxi Yongfu Lo-Han-Kuo Factory, 6/3/97)
- AC-4: Chemical Composition Analysis
- AC-5: Toxicity Studies (by Institute of Chinese Medicine, 2/20/92)

These 5 documents have been translated to English and split into 8 Sections (referred as AE-1 to AE-8) by NMI's previous translators. The translated package was reviewed by NMI and its regulatory consultant, CanTox. Fourteen questions on Package A were raised by CanTox to clarify some regulatory issues. These questions were translated into Chinese and send to the Chinese manufacturer of Lo-Han-Kuo extract. The response to these questions contains multiple Chinese and Japanese documents (referred as Package B). Sinotech was contracted to review this Package, to answer these 14 regulatory questions based on the Chinese documents provided in Package B, and to translate only those portions of Chinese documents in Package B needed to answer these questions.

Package B contains the following documents:

- Q&A: 14 Questions in English, the Chinese translation, and the answers in Chinese referring the following Attachments (3 pages)
- Attachment 1: Inspection records for 5 batches of extract, miscellaneous analysis of 2 batches (7 pages in Chinese)
- Attachment 2: Toxicity report of Lo-Han-Kuo extract (2 pages in Chinese)
- Attachment 3: Effects on immune response of Lo-Han-Kuo extract (3 pages in Chinese)
- Attachment 4: Assay and specifications of Lo-Han-Kuo extract (8 pages in Chinese)
- Attachment 5: Assay for total mogrosides in Lo-Han-Kuo (2 pages in Chinese)
- Attachment 6: Lo-Han-Kuo fruit listed in Chinese Pharmacopoeia (1 pages in Chinese)
- Attachment 6-1: Lo-Han-Kuo fruit listed in Chinese Herb Dictionary (3 pages in Chinese)
- Attachment 7: Pharmacological effects of Lo-Han-Kuo extract (1 pages in Chinese)

- Attachment 8: Study on the extraction process (2 pages in Chinese)
- Attachment 9: Marketing data of Lo-Han-Kuo fruit (1 pages in Chinese)
- Attachment 10: Assay of mannitol in Lo-Han-Kuo (2 pages in Chinese)
- Attachment 11: Assay of mogrosides in Lo-Han-Kuo (6 pages in Chinese)
- Attachment 12: Assay of grovenorine in Lo-Han-Kuo (3 pages in Chinese)
- Attachment 13: Study of sweeteners in Lo-Han-Kuo (6 pages in Chinese)
- Other information not directly related to Q&A: A Japanese vendor's technical information regarding a sweetener product containing Lo-Han-Kuo extract: (23 pages)

These Attachments in Package B contain 2 to 3 times more information than the 5 Chinese documents in Package A. However, some questions were not answered. A preliminary report was submitted by Sinotech to NMI and CanTox on 12/13/98 for their immediate reference. Sinotech wrote multiple letters to request the Chinese manufacturer to clarify the answers or provide more information. Based on the new information, a final report was completed here. Information between the "< >" sign were inserted by Sinotech for clarification.

Sinotech was also contracted to prepare the Material Safety Data Sheet (MSDS) for Lo-Han-Kuo Extract to its best capability using the currently available Chinese information in Package B. The MSDS is attached in Section B-3 of this document.

Section B-1: Certificate of Analysis

Question #1: "Certificates of analysis on 5 batches of extract to assess the variability within the limits <of> the <product> specifications."

Answer:

The "Inspection Records", equivalent to Certificate of Analysis (COA), of five batches of product were provided by the Chinese Manufacturer (Attachment 1). The assay Standard Operating Procedures (SOP) referred in the "Inspection Record" appeared to be what contained in Attachment 4 (c.f. Question #13).

Attachment 1 also contains other assay information for 2 batches. One data sheet reports the contents of crude fat (0.38%), total nitrogen (2.19%), organic carbon (61.05%), and crude fiber (0%) in the product sample of Batch #981016. The second data sheet reports the contents of carbon (53.13%), hydrogen (7.36%), and nitrogen (3.81%) in the product sample of Batch #980206.

The specification of the Lo-Han-Kuo Extract COA defines ~77% of the contents. The rest of ~23% material were not assayed routinely and were not defined in the COA. The assay results of these five COA cover 87%-90% of materials. The rest of 10%-13% material in the final product might be glucosides, fat, protein, pigment, ketone, etc.

Here is the translation of the COA on 5 batches:

Guilin Siter New Technology Company
Natural Botanical Product Factory
Product Quality Inspection Record

Product Name: Lo-Han-Kuo Glucosides
Inspection Date: 10/18/98
Batch Quantity: 5 Kg
Batch Number: 981016
Inspection Method: Standard Operating Procedures
Inspection Items: All items

Product Specifications and Inspection Results:

Items	Specifications	Inspection Results
Color	Yellow	Yellow
Appearance	Powder	Powder
Odor	Light Fragrance	Light Fragrance
Solubility	Easily dissolved in water or diluted ethanol	Passed
Glucoside Content	> 70%	82.3 %
Sweetness	>210 folds	215 folds
Water Content	< 6%	4.8%
Ash Content	< 1%	0.8%
Heavy Metal Ion Content	< 10 ppm	3 ppm

Inspected by: ID 303, ID 309
Approved by: ID 310
Date: 10/22/1998
Assay & Inspection Room
Guilin Siter New Technology Company, Natural Botanical Product Factory

Guilin Siter New Technology Company
Natural Botanical Product Factory
Product Quality Inspection Record

Product Name: Lo-Han-Kuo Glucosides
Inspection Date: 2/7/98
Batch Quantity: 100 Kg
Batch Number: 980206
Inspection Method: Standard Operating Procedures
Inspection Items: All items

Product Specifications and Inspection Results:

Items	Specifications	Inspection Results
Color	Yellow	Yellow
Appearance	Powder	Powder
Odor	Light Fragrance	Light Fragrance
Solubility	Easily dissolved in water or diluted ethanol	Passed
Glucoside Content	> 70%	81.5 %
Sweetness	>210 folds	215 folds
Water Content	< 6%	5.7%
Ash Content	< 1%	0.6%
Heavy Metal Ion Content	< 10 ppm	3 ppm

Inspected by: ID 304, ID 310
Approved by: ID 305
Date: 2/10/1998
Assay & Inspection Room
Guilin Siter New Technology Company, Natural Botanical Product Factory

Guilin Siter New Technology Company
Natural Botanical Product Factory
Product Quality Inspection Record

Product Name: Lo-Han-Kuo Glucosides
Inspection Date: 4/15/97
Batch Quantity: 100 Kg
Batch Number: 980410
Inspection Method: Standard Operating Procedures
Inspection Items: All items

Product Specifications and Inspection Results:

Items	Specifications	Inspection Results
Color	Yellow	Yellow
Appearance	Powder	Powder
Odor	Light Fragrance	Light Fragrance
Solubility	Easily dissolved in water or diluted ethanol	Passed
Glucoside Content	> 70%	85.7 %
Sweetness	>210 folds	215 folds
Water Content	< 6%	4.2%
Ash Content	< 1%	0.75%
Heavy Metal Ion Content	< 10 ppm	1 ppm

Inspected by: ID 304, ID 309
Approved by: ID 305
Date: 6/5/1997
Assay & Inspection Room
Guilin Siter New Technology Company, Natural Botanical Product Factory

Guilin Siter New Technology Company
Natural Botanical Product Factory
Product Quality Inspection Record

Product Name: Lo-Han-Kuo Glucosides
Inspection Date: 5/20/96
Batch Quantity: 300 Kg
Batch Number: 960518
Inspection Method: Standard Operating Procedures
Inspection Items: All items
Product Specifications and Inspection Results:

Items	Specifications	Inspection Results
Color	Yellow	Yellow
Appearance	Powder	Powder
Odor	Light Fragrance	Light Fragrance
Solubility	Easily dissolved in water or diluted ethanol	Passed
Glucoside Content	> 70%	81.5 %
Sweetness	>210 folds	215 folds
Water Content	< 6%	4.8%
Ash Content	< 1%	0.7%
Heavy Metal Ion Content	< 10 ppm	3 ppm

Inspected by: ID 304, ID 310
Approved by: ID 305
Date: 5/25/1996
Assay & Inspection Room
Guilin Siter New Technology Company, Natural Botanical Product Factory

Guilin Siter New Technology Company
Natural Botanical Product Factory
Product Quality Inspection Record

Product Name: Lo-Han-Kuo Glucosides
Inspection Date: 11/25/95
Batch Quantity: 100 Kg
Batch Number: 951120
Inspection Method: Standard Operating Procedures
Inspection Items: All items
Product Specifications and Inspection Results:

Items	Specifications	Inspection Results
Color	Yellow	Yellow
Appearance	Powder	Powder
Odor	Light Fragrance	Light Fragrance
Solubility	Easily dissolved in water or diluted ethanol	Passed
Glucoside Content	> 70%	82.3 %
Sweetness	>210 folds	220 folds
Water Content	< 6%	3.7%
Ash Content	< 1%	0.8%
Heavy Metal Ion Content	< 10 ppm	2 ppm

Inspected by: ID 304, ID 309
Approved by: ID 310
Date: 12/1/95
Assay & Inspection Room
Guilin Siter New Technology Company, Natural Botanical Product Factory

Section B-2: Residual Pesticide

Question #2: "Name of which pesticides that are applied on the crop and tree. Pesticide test results."

Answer:

The seedlings were applied with small amount of pesticides referred by the Chinese Manufacturer as "ester-likes-from-insect-propelling-Chrysanthemum", which is defined as pesticides extracted from the Chrysanthemum family such as permethrin, fenvalerate, tetramethrin, allethrin, cypermethrin, etc. The usage of pesticide depends on the degree of pest infection. The pesticide is usually sprayed between April and May, while Lo-Han-Kuo blooms in August and the fruits are harvested in October. There is no reason to expect there would be any pesticide in the product extracted from the fruits since the spray occurs months before the blooming season.

Section B-3: Material Safety Data Sheet

Question #3: "Material Safety Data Sheet <for the Lo-Han-Kuo Extract Product>"

Answer:

An MSDS for Lo-Han-Kuo Extract was prepared by Sinotech using information from the Chinese documents in Package B. The MSDS format was created based on the MSDS form suggested by U.S. Department of Labor and the guidelines set up by Office of Safety and Hygiene Administration (OSHA). The MSDS of chemicals or biochemical agents sold by several US companies were also used as a reference. A conservative approach was taken to prepare the MSDS. Multiple carcinogenic lists published by government offices have been checked to confirm that the active ingredients of Lo-Han-Kuo extract are not listed in these tables and are not considered as carcinogenic or toxic.

Here is the MSDS:

Nature's Marvel International

1681 Alta La Jolla Drive
La Jolla, CA 92037, USA
Emergency 1-619-456-4501
Fax 1-619-459-2428

MATERIAL SAFETY DATA SHEET MSDS NLE-1.3 1-19-99

SECTION 1 - PRODUCT IDENTIFICATION

Product Name: Lo-Han-Kuo Glucosides
Catalog #: NLE-001-10
Product Description: Extract of Lo-Han-Kuo fruits (Siraitia grosvenori, Swingle)
Appearance: Dry powder

SECTION 2 - COMPONENTS/INGREDIENT INFORMATION

Common Name: Mogrosides
Trade Name: to be determined
Composition: Mogrosides

SECTION 3 - HAZARDS IDENTIFICATION

Label Precautionary Statements:
Avoid inhalation. Inhalation may cause irritation or allergic reaction.
Avoid contact with skin and eyes.

SECTION 4 - FIRST AID MEASURES

In case of contact with eyes, immediately flush eyes with copious amounts of water for at least 15 minutes.
If ingested, rinse mouth with water followed by drinking water.
If inhaled remove person to fresh air. If allergic reaction occurs seek medical help.
In case of contact with skin, wash skin with soap and water.

SECTION 5 - FIRE FIGHTING MEASURES

Extinguishing media : Water spray, carbon dioxide, dry chemical powder or appropriate foam.
Combustion Products : Carbon monoxide and carbon dioxide
Special Fire-fighting Procedures : Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes or inhalation.

SECTION 6 - ACCIDENTAL RELEASE MEASURES

Wear respirator, rubber gloves, chemical safety goggles and protective clothing. Sweep up gently, minimizing the raising of particulate, place in bag and hold for waste disposal. Wash spill site with soap and water after material pickup is complete, then ventilate area.

SECTION 7 - HANDLING AND STORAGE

Storage: Store in tightly sealed containers. Although the product may be stored at room temperature, storage at 4 ∞C is recommended.
Handling : Do not handle without the proper safety equipment outlined in Section 8.

SECTION 8 - EXPOSURE CONTROLS/PERSONAL PROTECTION

Wear appropriate NIOSH approved respirator, chemical resistant gloves, safety goggles, and other protective clothing. Use only in a chemical fume hood.
Do not breathe dust.
Do not get in eyes, on skin, or on clothing.
Wash thoroughly after handling.

SECTION 9 - PHYSICAL AND CHEMICAL DATA

Physical state : Dry powder
Color: Ranges from yellow to light yellow
Percent Volatile: N/A
Vapor Pressure: N/A
Solubility in Water: N/A
Evaporation Rate: N/A
Odor : Light fragrance
Vapor Density: N/A
Specific Gravity: N/A
Melting Point: N/A
NFPA Rating: N/A
Flash point: N/A

SECTION 10 - STABILITY AND REACTIVITY

Stability: Stable
Incompatibilities: Strong oxidizing agents
Hazardous Decomposition Products: Thermal decomposition may produce carbon monoxide, carbon dioxide, nitrogen oxides and sulfur oxides.

SECTION 11 - TOXICOLOGICAL INFORMATION

Not listed as a carcinogen by the National Toxicological Program (NTP), the International Agency for Research on Cancer (IARC), or by OSHA. Not listed by the Registry of Toxic Effects of Chemical Substances (RTECS).

TOXICITY DATA
Oral-Rat LD50: > 24 mg/kg
Intravenous-Mouse LD50: N/A

SECTION 12 - ECOLOGICAL INFORMATION

No evidence of ecological toxicity, mobility, degradability, or bio-accumulation. Further data not yet available.

SECTION 13 - DISPOSAL CONSIDERATIONS

Combine material with combustible solvent and incinerate in a chemical incinerator which is properly equipped with afterburner and scrubber.

SECTION 14 - TRANSPORT INFORMATION

Contact Nature's Marvel International for transportation information.

SECTION 15 - REGULATORY INFORMATION

The product is not listed as a hazardous substance under 40 CFR section 302.4 pursuant to CERCLA; it is not listed as an extremely hazardous substance under Appendix A to 40 CFR 355; it is not listed as a hazardous waste under 40 CFR section 261, pursuant to RCRA; it is not listed in the 29 CFR part 1910, subpart Z list under OSHA; and is not considered a hazard under the List of Threshold Limit Values for Chemical Substances and Physical Agents in the Work Environment published by ACGIH. Other regulatory requirements are unknown at this time.

SECTION 16 - OTHER INFORMATION

The enclosed information is based on information Nature's Marvel International believes to be accurate, however; it should not be considered all inclusive, as it is to serve only as a guide. Proper handling should be exercised at all times. Nature's Marvel International shall not be held liable for any damage resulting from the handling or contact with this product.

Section B-4: Content Analysis for Food

Question #4: "Analytical method to verify the quantity of Lo-Han-Kuo <active ingredients> in food"

Answer:

This question is redundant to Question #11, as confirmed by CanTox on 11/30/98. Please refer to Section B-11 for details.

Section B-5: Usage before 1958

Question #5: "Report from Chinese government and/or the factory which shows the production of the extract and sales of extract before 1958"

Answer:

According to Attachment 9, Lo-Han-Kuo fruits had been used as a supplement of Chinese herb medicine before 1970, and the commercialization of Lo-Han-Kuo extract products was started from 1980 (c.f. Section B-8). The usage of Lo-Han-Kuo fruits before 1958 was described in Document AC-3, Certificate of Lo-Han-Kuo Glucosides as a Food Additive, but was not translated by NMI's previous translator. The related portions were translated here:

> "Lo-Han-Kuo has been used by Chinese for drink and medicine for more than 300 years. Its value as a natural sweetener and a herb medicine has been well recognized."
>
> "Lo-Han-Kuo is an unique herb only found at the south part of China, especially around Yongfu, Lingqua, Longshen areas at the north of Guangxi Province. As the production center of Lo-Han-Kuo, Guangxi Yongfu area produces approximately 70% of Lo-Han-Kuo in China."
>
> "According to the County History of Guangxi Yongfu, local people has cultivated Lo-Han-Kuo crops and collected the fruits for more than three hundred years. The Guangxi Chinese Medicine printed in 1963 described the detailed record of using Lo-Han-Kuo as a medicine in 1885 at Guangxi Yongfu."
>
> "According to the Guangxi Chinese Medicine, Lo-Han-Kuo is sweet, not toxic, beneficial to Lung and Spleen Channels <of Qi>. It can stop coughing, improve digestion, and serve as a refrigerant. It can be used to cure coughing, constipation, etc."

The historical record of the usage of Lo-Han-Kuo fruits by Chinese for at least 300 years can help justifying the GRAS status of Lo-Han-Kuo extract.

Section B-6: Pharmacopoeia Listing

Question #6: "A copy of the 1997 China Pharmacopoeia listing"

Answer:

The Lo-Han-Kuo section in the China Pharmacopoeia 1995 edition (Attachment 6) was translated below.

Important Notes: This Chinese document contains some Chinese medicine concepts which are very different to those of the Western medical science. Chinese medicine is based on "Qi" which is the internal energy flowing in the "Channels" of human body and can be felt, controlled, and enhanced by practicing "Qi-Gong". According to the Chinese medicine: when the "ying" and "yang" Qi are not balanced or the flow of Qi is blocked, illness will occur. To cure the illness, one must balance the ying and yang by herb medicine, acupuncture, practicing Qi-Gong, etc. The original description of Lo-Han-Kuo as a Chinese herb medicine in these documents can be very confusing to most Western scientists. To avoid raising more questions than answering them, Sinotech has translated the document in such a way which can be better understood by regular Western scientists. For example: the original statement of "It can put off the FIRE <i.e. too much yang Qi> in lung" was translated into "It can help the function of lung", which is not what the Chinese document literally said but is what it means, at least to Sinotech's best knowledge. Sinotech can translate it in a more literally "accurate" (but confusing) way if it is what NMI and CanTox prefer.

Here is the translation of Attachment 6:

China Pharmacopoeia, 1995 edition, page 185

Luo-han-kuo (Fructus Momordicae)

The Subject is the dried fruit of a cucurbitaceous plant, Lo-Han-Kuo (Momordica grosvenori Swingle). The fruit is harvested in the Fall when it turns from light green to dark green. The fruit is partially dried in the shade for several days and further dried by baking at a medium temperature.

[Appearance]: The Subject is oval or spherical with a height of 4.5 to 8.5 cm and a diameter of 3.5 to 6 cm. The surface is brown, light brown, or greenish brown with dark spots and yellow fuzz. It might have 6 to 11 strips, with residual style at the top and the scar of peduncle at the bottom. It is light and crispy. The (outer) pericarp is thin and can be easily broken. The pulp (i.e. middle and inner pericarps) is spongy and light brown. Numerous seeds are flat, circular, pink to brownish red with a length of ~1.5 cm and a width of ~1.2 cm. The seed is concave in the center with radial streaks on the side and a groove on the edge of the seed. The Subject has a light odor and is sweet.

[Identification]:

1. The powder product of this Subject is brown. The pericarp cells usually remain in groups, and are yellowish and in square or oval shape with diameters of 7-38 micrometers. The cell walls are thick with clear holes and grooves. The cells of seed skin are in rectangular or irregular shape with thin cell wall, holes and streaks on the surface. The fibers are in long shuttle shape with diameter of 16-42 micrometers, large cell chamber, clear holes on the cell wall. The xylem vessels contain step-wise or helical streaks. "Thin wall" cells are in irregular shapes with streaks.

2. Mix 2 g of the powder product with 20 mL of 50% ethanol. Heat for 30 minutes with vapor condensed and recycled. Filter the mixture and concentrate the filtrate to 5 mL by evaporation. Extract the concentrate with 10 mL and repeat with 5 mL of n-butanol. Completely evaporate the combined butanol solution and add 0.5 mL of methanol to dissolve the residual for assay. Repeat the above step using standard product to prepare the "control" sample for assay. Following the standard thin layer chromatography procedures (Appendix VI-B) to load 10 micro-liters of both methanol samples on the silicon-G plate and elute with chloroform:methanol:water (60:10:1). After elution and drying, spray the plate with 10% sulfuric acid / ethanol solution and dry with hot air until the color of spots is clear. The sample should have the same spot at the same location on the chromatogram comparing with the control.

[Characteristics]: Sweet and refrigerant. Beneficial to Lung and Intestine Channels <of Qi>.

[Functions]: It can help the function of lung and serve as a refrigerant. It can resolve constipation by improving the function of intestines. It can be used to cure hacking cough, throat pain, aphasia, constipation, etc.

[Dosage]: 9-15 g

[Storage]: Keep it dry. Prevent mold growing and insect biting.

Section B-7: Listing as Food and Medicine by Chinese Government

Question #7: "Copy of 1987 listing of Lo-Han-Kuo as a medicine or herb or dietary supplement by the Chinese Academy of Medicine of the Ministry of Hygiene"

Answer:

The 1987 listing of Lo-Han-Kuo as a health enhancing medicine by the Ministry of Hygiene, People's Republic of China is translated below. The information inside of the sign, < >, was added by Sinotech for clarification.

Regulation of Chinese Health Enhancing Medicine

Ministry of Hygiene, People's Republic of China
Document ID: Wei-Yao (87) #70
October 28, 1987

1. This regulation was formulated based on the "Drug Administration Law of People's Republic of China" to enforce the regulation of "Chinese health enhancing medicine" and to protect the health of Chinese people.

2. "Chinese health enhancing medicine" refers to the medicine which has definite benefits on enhancing human health or curing health problems, is nutritious, and has no toxic effect on human body even after being taken regularly for a long time.

3. "Chinese health enhancing medicine" must not contain medicine with toxicity, radioactivity, psychotic effect, or anesthetic effect. In general, it does not use materials from endangered animals, endangered plants, or importing materials as its major raw materials or components.

4. All additives or ingredients used for "Chinese health enhancing medicine" must follow the requirements and regulations for food and drugs.

5. The production and clinical trials of "Chinese health enhancing medicine" must be inspected and approved by the Office of Hygiene of each province, autonomous region, or city. The approval document must be submitted to the Ministry of Hygiene, with a approval ID following the format of "province-Wei-Yao-Chan (year)Z-number". For example: "Herpei-Wei-Yao-Chan (87)Z-01". The medicine approved by the Office of Hygiene of each province, autonomous region, or city will not be granted with a "Certificate of New Drug" and will not be entitled to the grace period of New Drug. To obtain the "Certificate of New Drug" for a "Chinese health enhancing medicine", the local Office of Hygiene must submit the documents to the Ministry of Hygiene for approval. The approval process will be identical to "Class III (Chinese Medicine) New Drug Application". The approval ID will follow the format of "(year)-Wei-Yao-Chan Z-number".

6. The application information package of "Chinese health enhancing medicine" must follow the requirement of "Class III (Chinese Medicine) New Drug Application". Since "Chinese health enhancing medicine" is usually taken on a routine basis for a long time, the safety issue must be the focus.

7. The production and management organizations of "Chinese health enhancing medicine" must be regulated following Chapter 2 and 3 of "Drug Administration Law".

8. The packaging, advertisement, labeling of "Chinese health enhancing medicine" must be regulated in the same way as "Therapeutic Medicine" following "Drug Administration Law".

9. The expenses of using "Chinese health enhancing medicine" should not be reimbursed by the government.

10. This regulation is effective immediately. Any violation of this regulation must be prosecuted according to "Drug Administration Law".

(First Batch)

Under Item #8 of "The Food Hygiene Law of People's Republic of China", the following listed materials are both food and medicine according to Chinese tradition:

A. Species listed in both "Pharmacopoeia of People's Republic of China, 1985 edition" and "Food Ingredients Table, 1981 edition #3" (excluding wild vegetables) published by the Institute of Hygiene of the Chinese Academy of Medicine.

B. The following items:

1. Wu-Shau snake <body of Zaocys dhumnades Cantor>
2. Viper <body of Agkistrodon halys Pallas>
3. Chinese jujube <seed of Zizyphus jujuba Mill. or Z. vulgaris Lamarck var. spinosus Bunge>
4. Oyster shell <shell of Ostrea gigas Thunb, O. rivularis Gould, O. talienwhanensis Crosse>
5. Gardenia <fruit of Gardenia jasminoides Ellis>
6. Licorice <root of Glycyrrhizae radix, G. uralensis, or G. glabra>
7. Dai-Dai flower <flower of Citrus aurantium L. var. amara Engl.>
8. **Lo-Han-Kuo** <fruit of Siraitia grosvenorii (Swingle) C. Jeffrey ex A.M. Lu et Z. Y. Zhang>
9. Cassia <bark of Cinnamomum cassia Presl>
10. Sickle senna <seed of Cassia tora L.>
11. Tsai-Lou-Zi <seed of Raphanus sativus L.>
12. Dried orange peel < fruit skin of Citrus reticulata Blanco>
13. Sha-Ren < fruit of Amomum longiligulare T. L. Wu>
14. Black plum <fruit of Prunus mume Sieb. et Zucc.>
15. Ro-Do-Kuo <seed of Myristica fragrans Houtt>
16. Angelica < root of Angelica dahurica Benth. et Hook>
17. Winter aster <flower of Chrysanthemum morifolium Ramat or C. indicum L.>
18. Bishop wort <plant of Agastache rugosa (Fisch. et Mey.) O. Ktze.>
19. Sha-Ji <fruit of Hippophae rhamnoides L. subsp. (yunnanensis, turkestanica, mongolica, or sinensis) Rousi>
20. Yu-Li-Ren <seed of Prunus humilis Bge or P. japonica Thunb.>

21. Chinese white olive <fruit of Canarium album raeusch>
22. Longstamen onion leaf <leaf of Allium macrostemon bge>
23. Peppermint<plant of Mentha haplocalyx Briq.>
24. Clove < flower bud of Eugenia aromatica Merr.l et Perry, E. caryophyllata Thunb., Syzygium aromaticum, or Caryophyllus aromaticus>
25. Kao-Lian-Jiang <seed of Sorghum vulgare pers>
26. White nut < nut of Ginkgo bilobal>
27. Xiang-Xiu <plant of Rabdosia rosthornii (Diels) Hara, Mosla chinensis Maxim., M. dianthera (Buch. Ham.) Maxim., Origanum vulgare L., Elsholtzia densa Benth, E. stanuntonii Benth., or E. eriostachya Benth>
28. Huo-Ma-Ren <fruit of Cannabis sativa I.>
29. Mandarine orange <skin of the fruit of Citrus tangeriana Hort. et Tanaka, C. erythrosa Tanaka, C. grandis Osbeck var. tomentosa Hort., or C. chachiensis Hort.>
30. Tuckahoe < fruit body of Poria cocos Wolff = Pachyma hoelen Rumph>
31. Xiong Yuan <fruit of Citrus wilsonii Tanaka>
32. Safflower <flower of Carthamus tinctorius L.>
33. Purple perilla <leave of Perilla frutescens Britton var. crispa Decaisne or P. frutescens Britton var. acuta Kudo>

Section B-8: Consumption

Question #8: "Any survey, report or scientific publications which estimates daily, weekly, monthly or yearly consumption of Lo-Han-Kuo"

Answer:

Attachment 9 (Journal of Chinese Medicine Information 1996(9)13) contains some marketing information about the consumption of Lo-Han-Kuo. Please note that the units are very different from what in the Western world. Some conversion factors are listed here:

- Weight: 1 "Jin" is 0.5 kilogram
- Area: 1 "Mu" is 0.1647 acre
- Currency: 1 "Yuan" in Ren-Min-Bi (RMB) is equivalent to approximately 1/8 US dollars.

Here is the translation of Attachment 9:

Journal of Chinese Medicine Information, 1996
Volume 3, Number 9, page 13

Market of Chinese Herb Medicine: Analysis of the Production and Sales of Lo-Han-Kuo

Lo-Han-Kuo, or Han-Kuo, is the dried fruit of a cucurbitaceous plant. It can help the function of lung and serve as a refrigerant. It can resolve constipation by improving the function of intestines. It is mainly produced in Guangxi, especially at the Yongfu and Lingqua areas, which are known as "the county of Lo-Han-Kuo". It is not only sued as a supplement of herb medicine, but also used as the major components of many over-the-counter Chinese medicine. It is also a traditional exporting item, among many other Chinese herb medicine. Lately, many local government offices have considered Lo-Han-Kuo as a major business. The production of Lo-Han-Kuo has increased very significant. The total cultivation area is over 10,000 "Mu" and the annual production volume is around 50-60 million "Jin". 1995 was a bumper year for Lo-Han-Kuo, with the annual production over 60 million Jin. Despite of the increase of production, the sale was even better than before and the price was doubled. The current price at production site of Lo-Han-Kuo fruit has increased from 0.62 Yuan in last year to 1.2 Yuan in this year for each large-size fruit; from 0.45 to 1.0 Yuan for each medium-size fruit; and from 0.26 to 0.5 Yuan for each small-size fruit. Based on the author's investigation, the price increase was due to the following reasons:

1. The medical value was confirmed and the usage volume increased: Before 1970, Lo-Han-Kuo had only been used in the Guangxi and Guangdong areas as a local Chinese herb medicine. It was seldom used in other areas. The annual production and sale volume was less than 10 million Jin. After the medial application of Lo-Han-Kuo was recorded in the 1991 edition of Pharmacopoeia of People's Republic of China, the application has been spread to the whole China and the medical effects have been confirmed again and again. With the confirmed medial value, Lo-Han-Kuo started to be sold in the whole China. Sine 1980, the sale volume increased year after year. In the 1990's, the sales increase dramatically. According to data gathered by government, the annual domestic sales were 5 million piece of fruits in 1970's, 15 million pieces in late 1980's, 25 million pieces lately, and over 30 million pieces in 1995.

2. The types of applications increased: For a long time before 1970's, Lo-Han-Kuo had only been used as a supplement of herb medicine. Since 1980, the medical usage of Lo-Han-Kuo has been increased with the growth of Chinese medicine industry. To date, there are over 20 over-the-counter Chinese medicine products and approximately 10 health products using Lo-Han-Kuo as the main raw material. There are more than 50% of domestic sales of Lo-Han-Kuo was as the raw material for the above mentioned products. In addition, Lo-Han-Kuo is also used to prepare drink. It is sweet, tasty, refrigerant, and known to be helpful

for the lung. The Lo-Han-Kuo drink is very popular and contributes to the sales of Lo-Han-Kuo.

3. The export volume increased: Lo-Han-Kuo is a traditional exporting item among many other Chinese herb. It is very welcomed by other countries. Since 1980, Lo-Han-Kuo and its over-the-counter medicine products have entered the Europe and America markets. According to related government offices, the export amount of Lo-Han-Kuo increases 2% per year. Annual export amount increases more than 6 folds, from 5 million pieces in 1970 to 35 million pieces of Lo-Han-Kuo fruit in 1995. Lo-Han-Kuo is mainly exported from Guangzo, Shanghai, and Tianjin

Due to the above mentioned reasons, the recent production volume of Lo-Han-Kuo cannot meet the demand of domestic and foreign markets, causing the price to increase. According to the market trends, we must increase production to fulfill the market demand by improving cultivation techniques and area productivity.

Section B-9: Composition Measured

Question #9: "On what basis is the chemical composition of Lo-Han-Kuo Extract standardized (Total Mogrosides? An individual Mogroside?)"

Answer: Total Mogrosides

Section B-10: Specifications

Question #10: "What are the chemical composition specification limits"

Answer:

The chemical composition specifications have been provided and translated in the 5 COA under Question #1.

Section B-11: Assay Methods and Validation

Question #11: "What method (complete detailed steps) is used to measure the amount of Lo-Han-Kuo extract in foods to which it has been added and what procedure is used to validate the method (details)"

Answer:

The Chinese manufacturer provided a procedure for measuring the amount of Lo-Han-Kuo extract in foods (referred as "Lo-Han-Kuo Products" by the Chinese manufacturer) to which it has been added. The information is translated below:

Assay Methods for the Contents of Lo-Han-Kuo Glucosides in Lo-Han-Kuo Products

China Guilin Siter New Technology Company
Natural Botanical Product Manufacturer
1-4-1999

1. Instrument and Reagents

1.1 UV Spectrometer
1.2 Electronic Balance (1/100,000 sensitivity)
1.3 Lead acetate
1.4 Methanol
1.5 Ethanol, anhydrous
1.6 Vanillin
1.7 Sulfuric Acid
1.8 Standard sample of Lo-Han-Kuo Extract

2. Establishment of Standard Curve

Weigh exactly 30 mg of Lo-Han-Kuo Extract Standard and add into a 10 mL volumetric bottle. Add 70% ethanol to dissolve and Q.S. to the final volume<of 10 mL>. Mix well and pipette exactly 25, 50, 75, 100, 125 microliters of solution to 10 mL test tubes with sealing caps. Add 70% ethanol to each tube and Q.S. to 0.5 mL. Add 0.5 mL of 10% vanillin - ethanol solution to each tube. Mix well and sit the tubes in ice bath. Add 5 mL of 75% sulfuric acid solution and mix well. Heat the tubes to 50 C for 20 minutes then put tubes back to the ice bath immediately. After 10 minutes in ice bath, measure the absorbence at 530 nm, which is the wave length of peak absorbence. Plot the amount of Standard vs. the absorbence to establish the Standard Curve.

3. Determination of the Content <of Lo-Han-Kuo Extract> in the Sample

3.1 Sample preparation:

Weigh exactly 20 g of Lo-Han-Kuo product and suspend with distilled water. Transfer the suspension into a 250 mL flask. After soaking for 10-15 minutes, add 2-5 mL of neutralized lead acetate to remove protein. Mix well for 10 minutes and add water to 150 mL. Filter to remove precipitate. Rinse the precipitates with 30 mL of water for 3 times (90 mL total amount of water)Combine all filtrate solutions. Add the filtrate to a 500 mL round bottom bottle and concentrate until it is dry by rotating film evaporation. Add 100 mL of methanol and 5 gram of anhydrous sodium sulfate and extract the dried material by heating with reflux condensation for 1 hour. Filter the extraction suspension after cooling and repeat

the above methanol-sodium sulfate extraction process for a total of three times. Combine three filtrates and concentrate by a K-D concentrator until it is dry.

3.2 Measurement of Lo-Han-Kuo glucosides

Add 70% ethanol to dissolve the dry material in the K-D concentrator and remove the solution to a 10 mL volumetric bottle. Add 70% ethanol and Q.S. to the final volume of 10 mL. Mix well and pipette exactly 75 microliters of solution to a 10 mL test tube with sealing caps. Follow the steps in Section 2 to measure the absorbence. Use the Standard Curve to convert the absorbence to the equivalent amount of Standard in microgram, "C". The Content <of Lo-Han-Kuo Extract> in the Sample can be determined by the following equation:

$$\text{Content in Sample (\%)} = C / (7.5 \times W) \times 100\%$$

where C = the amount obtained from the Standard Curve, micro-gram
W = the amount of Sample, mg

Section B-12: Chemical Abstract System Registry Number

Question #12: "Have chemical abstract system registry numbers been assigned to Lo-Han-Kuo or any of it's constituents (Mogrosides, etc.). If so, please indicate those numbers."

Answer:

The chemical abstract service registration numbers of mogrosides were identified by Sinotech:

Mogroside IV: 89590-95-4
Mogroside V: 88901-36-4
Mogroside VI: 89590-98-7

Section B-13: Assay Standards

Question #13: "What is the source, chemical composition and method of standardization for Lo-Han-Kuo "Standard" used in chromatographic and other analysis <at the Manufacturer's site>"

Answer:

Attachment 4 is the SOP of Guilin Siter Factory which describes the assay and standard used to generate the COA of 5 batches provided for Question 1. It contains most information needed to answer this Question:

- Method of Standardization: See Item 4.2.2 of the SOP (Attachment 4) for details.
- Chemical composition of Standard: Lo-Han-Kuo mogrosides, etc. (c.f. Item 3.2 of the SOP)
- Source of Standard: Lo-Han-Kuo Extract produced in Guilin Siter Factory (c.f. Item 4.2.1.6)

Here is the translation of Attachment 4:

Guilin Siter New Technology Company
Natural Botanical Product Manufacturer
Standard Operating Procedures

Q/TRP002-1995

Lo-Han-Kuo Glucosides

Guangxi Zuang Tribe Autonomous Region
Technology Inspection Bureau
Standard Operating Procedures Filing & Registration Stamp
#450000

Publishing Date: 1995-07-10 Effective Date: 1995-08-05
Published by: Guilin Siter New Technology Company, Natural Botanical Product Factory

1.0 Scope and Contents

This Standard Operating Procedures (SOP) apply to the Lo-Han-Kuo Glucoside Powder extracted and purified from fresh or dried Lo-Han-Kuo fruits.

2.0 <Reference> Procedures Cited

GB 5009.3	Assay Procedures for Water Content in Food
GB 5009.4	Assay Procedures for Ash Content in Food
GB 8451	Assay Procedures for Heavy Metal Content in Food
GB 8450	Assay Procedures for Arsenic Content in Food Additives
GB 7718	General Standards for Food Labeling

3.0 Specifications

3.1 Appearance Specification

3.1.1 The product should be light yellow or yellow powder and very soluble in water and ethanol.

3.1.2 The product should have the unique fragrance of Lo-Han-Kuo and is very sweet.

3.2 Physical Property Specifications

Physical Property Specifications of Lo-Han-Kuo Extract

Item	Grade 1 Specification	Grade 2 Specification
Glucosides	> 85 %	> 75 %
Sweetness	240	210
Ash	< 2.0 %	< 2.2 %
Water	< 5 %	< 6 %
Lead (Pb)	< 0.002 %	< 0.002 %
Arsenic (As)	< 0.0001 %	< 0.0001 %
Infra Red Characteristic Peaks (wave number) +/- 10 cm^{-1}	3430, 1651, 1454, 1377, 1166, 1075	3430, 1651, 1454, 1377, 1166, 1075

4.0 Assay Methods

Unless noted differently, only distilled water or equivalent is used. All reagents are "analysis" grade.

4.1 Sensational Inspection

Put the sample on white paper and inspect it under appropriate natural light. It should be in light yellow or yellow color with the unique fragrance of Lo-Han-Kuo. Dissolve the sample in water to 1% concentration, the solution should taste very sweet.

4.2 Quantitative Analysis

4.2.1 Instrument and Reagents

4.2.1.1 UV Spectrometer
4.2.1.2 Electronic Balance (1/100,000 sensitivity)
4.2.1.3 Ethanol, anhydrous
4.2.1.4 Vanillin
4.2.1.5 Sulfuric Acid
4.2.1.6 Standard sample of Lo-Han-Kuo Extract -- Provided by this Factory. Store the Standard in a brown sealed bottle and place the bottle in a desiccator. Replace the Standard every other year.

4.2.2 Establishment of Standard Curve

Weigh exactly 30 mg of Lo-Han-Kuo Extract Standard and add into a 10 mL volumetric bottle. Add 70% ethanol to dissolve and Q.S. to the final volume of 10 mL. Mix well and pipette exactly 25, 50, 75, 100, 125 microliters of solution to 10 mL test tubes with sealing caps. Add 70% ethanol to each tube and Q.S. to 0.5 mL. Add 0.5 mL of 10% vanillin - ethanol solution to each tube. Mix well and sit the tubes in ice bath. Add 5 mL of 75% sulfuric acid solution and mix well. Keep

the tubes in 50 C water batch for 20 minutes then put back to the ice bath immediately. After 10 minutes in ice bath, measure the absorbence at 530 nm, which is the wave length of peak absorbence. Plot the amount of Standard vs. the absorbence to establish the Standard Curve.

4.2.3 Determination of the Content <of Lo-Han-Kuo Extract> in the Sample

Weigh exactly 30 mg of Sample and add into a 10 mL volumetric bottle. Add 70% ethanol to dissolve and Q.S. to the final volume of 10 mL. Mix well and pipette exactly 75 microliters of solution to a 10 mL test tube with sealing caps. Follow the steps in Section 4.2.2 to measure the absorbence at 530 nm. Use the Standard Curve to convert the absorbence to the equivalent amount of Standard in microgram, "C". The Content <of Lo-Han-Kuo Extract> in the Sample can be determined by the following equation:

$$\text{Content in Sample (\%)} = C / (7.5 \times W) \times 100\%$$

where C = the amount of Standard obtained from the Standard Curve, micro-gram
W = the amount of Sample, mg

4.3 Determination of Sweetness

Weigh 2 g of sucrose and dissolve in 100 mL of distilled water to prepare a 2% solution. Weigh 2 g of Sample and also prepare a 2% solution. Dilute the 2% Sample solution for 210 folds with distilled water. Compare the dilution Sample solution with the sucrose solution <by tasting it>. When the sweetness of these two solutions are equivalent, the dilution factor <of the 2% Sample solution> is the 'Sweetness" of the Sample.

4.4 Determination of Water Content

Follow Procedure GB5009.3

4.5 Determination of Ash Content

Follow Procedure GB5009.4

4.6 Determination of Heavy Metal (based on lead, Pb) Content

Follow Procedure GB8451

4.7 Determination of Arsenic Content

Follow Procedure GB8450

4.8 Infra Red Spectrum

4.8.1 Principle:

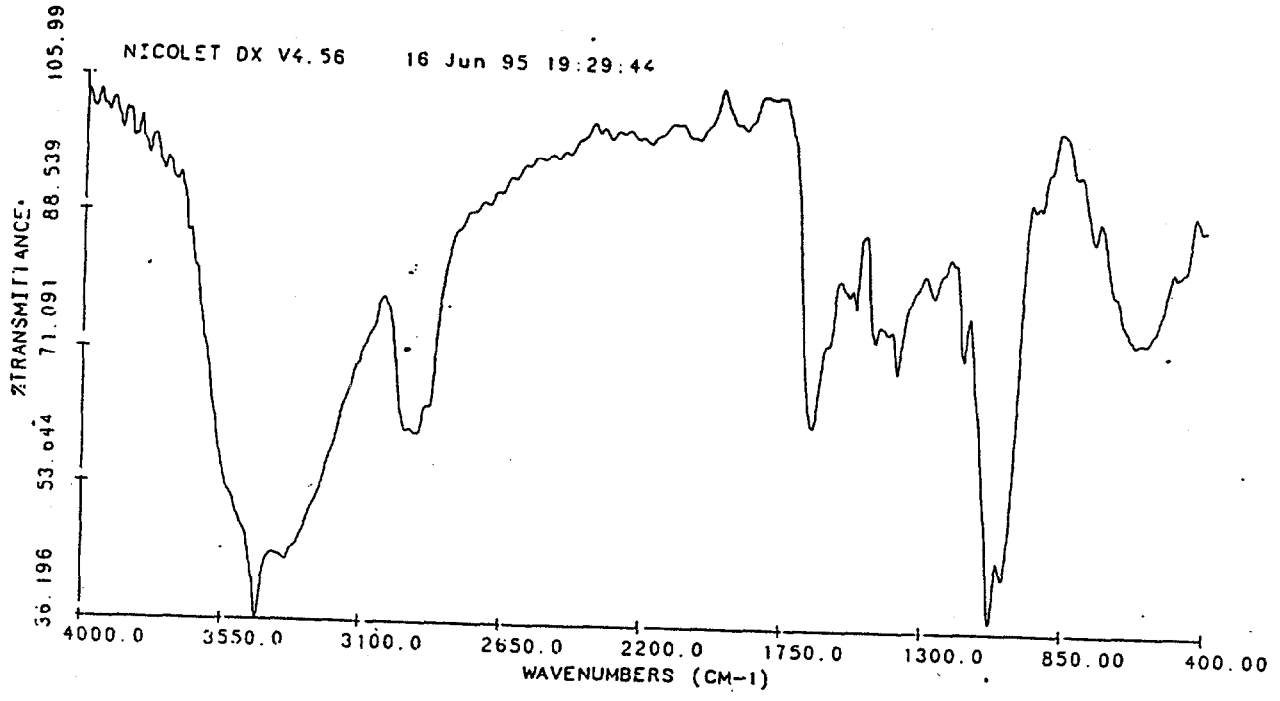
Mix the Sample with potassium bromide and press into a chip for Infra Red spectrum measurement. Compare the characteristic peaks on the spectrums of the Sample and the Standard.

4.8.2 Instrument:

Infra Red Spectrophotometer

4.8.3 Determination of the Wave Numbers of Infra Red Spectrum

Following the standard "Potassium bromide press chip method" of Infra Red Spectrum Measurement, mix the Sample with potassium bromide in a ratio of 1:100. Press the mixture into a chip and measure the Infra Red Spectrum. The Wave Numbers of the Sample spectrum should be consistent with what listed in the Physical Property Specification Table (Section 3.2). The Spectrum of Standard is attached in the following figure:



5.0 Inspection Guidelines

5.1 The product should be inspected by the Technical Inspection Department of the production factory. All products released from the factory must meet the specifications listed in this SOP. Each batch of product released from the factory must be accompanied with a Certificate of Quality Assurance confirming that the quality meets the specifications.

5.2 Take samples from more than 10% of packages in each batch, or at least 3 packages, whichever is larger. Remove 10 grams of sample from each package and mix all samples together. Divide the mixed sample into four groups and take 30 grams to put into two clean polyester bags. Put the each bag into another complex aluminum bag and seal. Label the bags with product name, batch number and sampling date. Take one bag for assay and another as the archive sample.

5.3 Every batch must be inspected against the Physical Property Specifications.

5.4 If the inspection results indicate that there is one item does not meet the specification, the inspection of the failed item can be repeated to determine the fate of this batch. If the repeated inspection still fails to meet the particular specification, the particular batch fails to meet the specifications.

5.5 When the customer and the factory disagree on the quality of product, both parties may negotiate or authorize an arbitrate organization to conduct the inspection according to this SOP.

5.6 Any product quality issues caused by inappropriate shipping or storage should not be the responsibility of the factory. It should be the responsibility of the organization handling shipping and storage.

6.0 Packaging, Labeling, Shipping, and Storage

6.1 Product should be first packaged in a polyester bag then packaged in another complex aluminum bag. The external package is a carton box with water-proof coating.

6.2 The label on the polyester bag should follow the guideline in GB7718.

6.3 A Certificate of Quality Assurance must be accompanied with the package. The Certificate should contain the following information: production factory, product name, address, batch number, net weight, production date, product specification ID number.

6.4 The external package must contain a robust label containing the following information: production factory, product name, address, batch number, net weight, production date, expiration date.

6.5 During the shipping process, the product should be kept from moisture, rain, over-pressure, sun light, heat, and contamination of toxic materials.

6.6 The product should be stored in a cool and dry shady area. It should be segregated from toxic, stink, or chemical materials.

6.7 The expiration date is two years from the production date.

Note: The SOP was drafted and published by Guilin Siter New Technology Company, Natural Botanical Product Factory.

SOP Originator: Lee Jin

Section B-14: Siamenoside

Question #14: "What is the sweetness of siamenoside triterpene and how much of occurs in the extract"

Answer:

A paper published by several researchers at Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences reviewed studies on chemical principles and uses of natural non-sugar sweeteners from Lo-Han-Kuo (Research and Development of Natural Products, 1992(1)72, Attachment 13). According to the paper, the sweetness of 0.01% siamenoside I solution is 563 folds of 5% sucrose solution, and the yield of siamenoside I is 0.047%. The yield is presumably based on the dry weight of Lo-Han-Kuo fruit but it was not defined in the paper. There are no information in this paper regarding the amount of siamenoside I in Lo-Han-Kuo extract to answer the second part of Question #14. However, Package A stated that the yield of Lo-Han-Kuo extract is 1% of dried Lo-Han-Kuo fruits. Therefore, the content of siamenoside I in Lo-Han-Kuo extract should be around 4.7%. Since the sweetness of siamenoside I is ~280,000 (i.e. 500 x 563) folds of pure sucrose, the sweetness of Lo-Han-Kuo extract should be at least 13,000 (i.e. 280,000 x 4.7%) folds of sucrose, which is dramatically inconsistent with the measured sweetness of Lo-Han-Kuo extract, around 200 folds of sucrose. The above inconsistency suggests that these data are questionable.

The sweetness of siamenoside I was also mentioned in Package A but the concentration of siamenoside for sweetness measurement was translated incorrectly by the previous translator. The siamenoside solution was diluted for 10,000 folds instead of 1,000,000 folds when its sweetness was compared with 5% sucrose solution. An English scientific paper was cited as the original source of the sweetness information (c.f. R. Kasai et al, 1989; Agric. Biol. Chem., 53(12):3347). However, upon reviewing the cited paper, no information about the sweetness of siamenoside was found.

It is interesting to note that three other glucosides from Lo-Han-Kuo are also extremely sweet, according to Attachment 13. The table from Attachment 13 containing related sweetness information was enclosed. Again, these data are questionable since the sweetness of Lo-Han-Kuo extract calculated from these data is much too high.

Here is the table with the sweetness and yield data in Attachment 13:

表1　罗汉果中的三萜成分

Name	Formula	m·p·(C)	[α] D	Yield(%)	水中的浓度与甜度*	References
mogroside IV (1)	$C_{54}H_{92}O_{24}\cdot 2H_2O$	185—188(dec)	−4.2 −5.8	0.12	0.012% 392	10, 11
mogroside V (2)	$C_{60}H_{102}O_{29}\cdot 2H_2O$	197—201(dec)	−11.7	0.029—1.0	0.012% 425	10, 11
siamenoside I (3)	$C_{54}H_{92}O_{24}\cdot 7/2H_2O$	—	+4.0	0.047	0.01% 563	10, 11
11-oxo-mogroside V (4)	$C_{60}H_{100}O_{29}\cdot 7/2H_2O$	—	+20.5	0.18	0.05% 84	8, 11
mogroside II E (5)	$C_{42}H_{72}O_{14}$	—	+35.2		tastless	11
mogroside III E (6)	$C_{48}H_{82}O_{19}$	—	+4.5		tastless	11
mogroside III (7)	$C_{48}H_{82}O_{19}$		+2.5		tastless	11
mogrol (8)	$C_{30}H_{52}O_4\cdot H_2O$	135—135.5	+70.0		tastless	9, 11
11-oxo-mogrol (9) (bryoducosigenin)	$C_{30}H_{50}O_4\cdot 1/2H_2O$				tastless	9, 10
mogroside VI (10)	$C_{66}H_{112}O_{34}$	198—204	−4.2		0.01% 125	8

*甜度以5%的蔗糖溶液为1计。

REPORTS ON THE TOXICOLOGY TESTS OF LUOHANGUO EXTRACTS (GLYCOSIDES) FOR EXPORT TO NORTH AMERICA

Nov. 15, 1996

Acadamy Institute: Department of Pharmacology, Guilin Medical College
Address: 20 Lequn Rd
Guilin, Guangxi 541001
P. R. China

Coordinators: Nanhai Goldstar Industrail CO. LTD.
Natural Product Factory, Guilin S&T New Technology Company.

Date of test: Aug. 13, 1996——Nov. 11, 1996

Ⅰ. Extract Samples

Five samples tested from five batches of the products (batch No: 951120, 960116, 960206, 960410, 960518) were provided by the Natural Plant Product Factory, Guilin S&T New Technology Company. All samples from the five batches show a slightly brawn colour (for quality examination results of the five batches of produced, see Apendix Ⅰ)

Ⅱ. Sample Treatments

Upon administration to the dogs each time, extract sample was dissolved in distilled water to make a solution of 100ml containing 30g of the extract. Sample of each batches was used for 18 days.

Ⅲ. Methods

1. Animal housing and feeding

Sixteen hybrid dogs weighting 8.0～9.0 Kg, age of 24～30 weeks, with eight males and eight females, were perchased from the local farmer market, guarantines proved all healthy. Each dog was assigned to a number, from 1 to 16, and beared a metal card with the number assigned. Dogs were randomly divided into groups, eight dogs per group, with No. 1, 5, 6, 8, 9, 11, 13, and 15 in the experimental group, No. 2, 3, 4, 7, 10, 12, 14 and 16 in the control group. Animals were housed individually in a steel cage (80×60×80cm) at

the Experiment Animal House of Guilin Medical College, with room temperature of 22±2 C, relative humidity of 30~40% and 12 hour dark-light cycle. The animals were fed with cooked rice as main food, three times a day supplement with cooled pork meat or/and fish and vegetables. Distilled water was provided as drinking water. (for water analysis results, see Supplement Document 2)

2. Luohanguo Extract Dose and Administration

Before starting experiment, dogs housed under the condition described above were allowed to acclimate the envirdment for four days. After 12 hour fasting, dogs of experiment group were given Luohanguo extract 3..0g/kg (BWT), (in 5ml/kg), twice daily at 8 : 00~8 : 30 and 17 : 00~17 : 30 by gavage (tube feeding) for consecutive 90 days; control group were given distilled water by the same method, during the 90 days of the Luohanguo extract feeding period, all animals were observed for their food and water intake, urine and stool excretion, and general behavior changes (if any). Each dog was weighed once a week and body weight was recorded, meanwhile, blood sample was drawn and urine sample was collected for laboratory analysis. At the 91th day (the second day of discontinue of Luohanguo extract feeding) all dogs in both groups were killed and organs were disected for pathological examinations.

3. Examinations

(1) Hematology: RBC, WBC, HB;

(2) Blood Biochemistry; blood K^{-}, P^{4-}, Cl^{-}, Ca^{2+};

(3) Liver function: total protein (TP), albumin (ALB), globubmin (GLO), ALT, AST;

(4) Renal function: urine volume, urine PH, BUN, Creatinine (Cr), urine protein;

(5) Blood sugar and urine sugar;

(6) pathological examinations of heart, liver, spleen, lung and kidney.

4. Statistic analysis

All result data was statistically analysized by one way of ANOVA, a $p<0.05$ was considered significat difference.

Ⅳ. Results

All results are shown in the tables 1 to 6.

Ⅴ. Discussions and Conclusions

There was no evidence showing effect of Luohanguo extract, 3.0g/kg daily (equivatent to 360 folds of the dose for a human adult) for 90 consecutive days on the animals body weights, food consumption and, urine and feces secretions. As seen from the Tables A to G, statistical analysis also shows no effects on blood biochemistries, urine chemical and functions of liver, kidney as well as on the histology of organs examined.

Investigators: Zhunian Tang, Qin Xu, Yuxian Wei, Xiao Jian Su .

P. I: Xiao Jian Su

桂林医学院实验记录

8只狗子均值

Experiment Data Sheet

汉果提取物(甜甙)3 g/kg 白连续灌服 90 天 8 只狗(第 1,5,6,8,11,13,15,号狗)各项检查结果统计表

Statistic calculations of the results of Examinations of the eight dogs(No. 1, 5, 6, 8, 9, 11, 13, 15) fed with Luohan-guo(glycosides) for consecutive 90 days.

Tab.A.B.

指标 Index	动物数 (n)	日期(天) Date											
		0	7	14	21	28	35	42	49	56	63	77	90
体重(kg) Body Weight	8	8.39±0.30	8.50±0.24	8.61±0.22	8.83±0.25	9.05±0.24	9.36±0.87	9.60±0.86	9.81±0.89	10.11±1.02	10.36±1.08	10.60±1.15	10.95±1.34
外观毛色 Hair Appearance	8	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
活动情况 Active(Condition)	8	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
心率(次/分) Heart Rate(time/min)	8	167.75±16.37	170.25±11.12	170.38±11.21	171.38±15.13	173.25±18.23	169.63±14.89	168.88±12.33	168.75±18.05	173.00±13.16	173.88±11.10	170.25±17.51	171.75±14.21
血压(mmHg) Blood Presure	8	121.12/72.38 ±5.74/±7.56	119.88/70.88 ±6.62/±7.99	126.38/72.00 ±10.46/±6.44	128.13/76.75 ±13.10/±7.92	123.38/72.88 ±8.28/±8.58	121.75/76.25 ±9.65/±8.03	120.13/71.63 ±8.63/±7.52	127.50/74.13 ±7.78/±5.36	125.83/72.00 ±9.95/±4.81	125.88/73.00 ±5.77/±7.63	124.38/72.63 ±7.33/±7.31	124.63/72.38 ±6.02/±7.86
呼吸(次/分) Respiration(time/min)	8	29.50±3.96	29.13±3.04	31.38±4.63	31.63±5.26	31.63±3.96	32.25±3.65	31.25±5.47	31.38±4.24	31.13±5.87	29.00±3.78	31.13±4.58	30.38±3.74
大便 Stool	8	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
小便 Urine	8	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
RBC:×10⁶/mm³	8	6.55±0.45	6.39±0.26	6.53±0.46	6.29±0.27	6.44±0.49	6.62±0.67	6.53±0.66	6.28±0.75	6.34±0.73	7.05±0.57	6.31±0.58	6.84±0.50
WBC:×10³/mm³	8	10.25±0.54	10.83±1.10	11.02±0.52	10.96±1.34	11.66±0.99	11.59±1.30	10.84±1.13	11.14±0.77	10.77±0.71	11.23±1.26	11.15±1.20	11.58±0.74
Hb: g%	8	13.13±0.39	12.31±0.73	12.78±1.04	12.45±0.33	13.14±0.64	12.64±0.72	12.71±0.79	12.41±0.59	12.39±0.42	12.31±0.45	12.49±0.72	12.53±1.12

A: General Condition

B: Hematology examination

Q: Normal

桂林医学院实验记录

Experiment Data Sheet

罗汉果提取物(甜甙)3 g/kg/日连续灌胃 90 天 8 只狗(第 1,5,6,8,11,13,15,号狗)各项检查结果统计表

Statistic calculations of the results of Examinations of the eight dogs(No. 1, 5, 6, 8, 9, 11, 13, 15) fed with Luohan-guo(glycosides) for consecutive 90 days.

Tab.F.

指标 Index		动物数 (n)	日期(天) Date											
			0	7	14	21	28	35	42	49	56	63	77	
血液生化检验F	血钾(mmol/L) Blood K^+	8	4.22±0.08	4.19±0.06	4.11±0.09	4.21±0.09	4.32±0.12	4.30±4.29	4.25±0.19	4.20±0.08	4.19±0.09	4.49±0.43	4.33±0.20	4.3
	血磷(mmol/L) Blood P^{+1}	8	1.73±0.06	1.79±0.05	1.74±0.07	1.75±0.06	1.70±0.17	1.35±0.10	1.88±0.13	1.90±0.13	1.77±0.13	1.75±0.08	1.70±0.10	1.76
	血氯(mmol/L Blood CL^-	3	112.6±12.13	109.2±7.37	113.3±14.1	114.2±14.3	112.1±10.40	103.2±31.3	123.4±22.7	116.6±7.89	114.0±10.7	121.4±18.2	108.8±11.7	116.9
	血钙(mmol/L) Blood Ca^{2+}	8	1.65±0.12	1.74±0.13	1.71±0.21	1.87±0.13	2.09±0.19	2.04±0.25	1.72±0.26	1.67±0.09	1.74±0.14	1.85±0.17	1.75±0.26	1.86

F: Blood biochemistry examination

Tab. G.

器官 Organs	动物数 (n)	重量(g) Weight	外观 Appearance	浊肿 Turbid swelling	空泡变性 Vacuolar degeneration	炎症灶 Inflammatory focus	坏死灶 Necrotic focus
肝 Liver	8	354.4±12.16	无异常 without abnormer	无 None	无 None	无 None	无 None
心 Heart	8	101.7±7.20	无异常 〃	无 〃	无 〃	无 〃	无 〃
肾 kidney	8	42.4±3.42	无异常 〃	无 〃	无 〃	无 〃	无 〃
肺 Lung	8	228.1±17.08	无异常 〃	无 〃	无 〃	无 〃	无 〃
脾 Spleen	8	63.3±3.63	无异常 〃	无 〃	无 〃	无 〃	无 〃

G: Organ's pathology examination

桂林医学院实验记录

Experiment Data Sheet

罗汉果提取物(甜武)3 g/kg/日连续灌服 90 天 8 只狗(第 1,5,6,8,11,13,15,号狗)各项检查结果统计表

Statistic calculations of the results of Examinations of the eight dogs(No. 1, 5, 6, 8, 9, 11, 13, 15) fed with Luohan-guo(glycosides) for consecutive 90 days

Tab.C.D.E.

	指标 Index	动物数 (n)	日期(天) Date 0	7	14	21	28	35	42	49	56	63	77	90
肝功能检验 C	TP (g/L)	8	58.15±5.20	58.88±5.10	57.20±6.18	56.90±2.35	58.88±4.82	58.23±6.18	57.80±5.35	55.38±5.14	58.10±2.36	62.16±1.24	62.40±4.94	58.46±
	ALB(g/L)	8	26.89±1.23	27.10±0.81	26.00±2.46	27.25±2.80	26.22±1.47	27.92±2.48	26.68±2.27	28.34±2.81	27.11±1.42	27.81±1.32	28.12±3.17	29.01±
	GLO(g/L)	8	32.25±3.47	32.24±1.10	30.04±2.85	28.10±4.38	30.27±2.79	30.94±4.07	33.40±6.18	31.62±19.67	29.48±1.33	33.40±2.01	30.72±2.72	30.50±
	ALT(u/L)	8	26.83±3.49	27.14±3.13	30.62±5.02	27.20±2.05	27.94±3.81	29.10±5.92	27.33±4.48	27.05±2.79	27.21±2.64	28.97±1.59	28.73±1.75	28.76±
	AST(u/L)	8	28.21±3.58	30.58±5.71	28.71±4.39	31.00±3.41	27.12±28.08	29.03±2.11	27.89±3.49	27.17±2.21	29.68±1.78	28.62±3.01	31.17±3.64	30.23±
肾功能检验 D	尿量(ml/日) Urine Volume(ml/day)	8	211.25±61.15	239.00±28.60	217.63±22.69	210.50±43.12	212.00±40.60	280.38±61.76	210.00±31.80	227.25±24.18	235.63±20.45	208.25±60.18	202.63±38.45	200.25
	尿液 PH Urine PH	8	6.15±0.23	6.14±0.11	5.94±0.38	6.02±0.07	6.00±0.45	6.20±0.26	5.93±0.41	6.23±0.22	6.23±0.16	6.16±0.40	6.08±0.63	6.33±
	BUN(mmol/L)	8	3.40±0.33	3.36±0.35	4.08±0.49	3.33±0.25	3.31±0.46	3.83±0.64	3.87±0.43	4.23±1.00	4.06±0.67	3.93±0.34	4.07±0.80	3.83±
	Cr(mmol/L)	8	77.80±11.84	79.19±6.47	81.25±10.15	73.41±9.02	77.38±5.83	70.38±8.26	75.16±5.83	72.97±3.38	75.69±6.30	73.14±6.22	76.17±3.81	74.57±
	红蛋白 (g/L) Red Protein	8	0	0	0	0	0	0	0	0	0	0	0	0
血尿糖检验 E	血糖 (mmol/L) Blood sugar	8	5.44±0.81	5.23±0.69	5.19±0.67	5.23±0.33	5.12±0.50	5.53±0.60	5.07±0.21	5.06±0.46	5.39±0.63	5.29±0.45	5.23±0.32	5.21±
	尿糖 (mmol/L) Urine Sugar	8	0	0	0	0	0	0	0	0	0	0	0	0

C: Liver function examination .

D: Kidney function examination .

E: Blood sugar and urine sugar examination

一只的咽拔去8个

桂林医学院实验记录

Experiment Record of Guilin Medical College

(1)号狗试验记录表: Experiment Record Table of dog(1)

No. 1

	指标 Index	日期(天) Date											
		0	7	14	21	28	35	42	49	56	63	77	9
一般情况A	体重(kg) Body Weight	8.5	8.7	8.7	9.1	9.5	11.0	11.7	12.0	12.6	13.0	13.4	14.
	外观毛色 Hair Appearance	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q
	活动情况 Aative(Condition)	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q
	心率(次/分) Heart Rate(time/min)	186	175	182	190.	187	179	183	191	176	183	190	186
	血压(mmHg) Blood Presure	120/80	128/82	119/78	124/84	117/80	130/90	126/86	118/76	116/74	124/76	130/80	126/8
	呼吸(次/分) Respiration(time/min)	34	28	36	31	29	34	28	35	36	27	31	30
	大便 Stool	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q
	小便 Urine	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q
血液学检验B	RBC: $\times 10^6$/mm^3	6.34	5.76	6.47	7.23	6.75	7.34	6.58	5.92	6.87	7.35	6.92	7.29
	WBC: $\times 10^3$/mm^3	10.44	9.65	11.23	8.95	10.51	8.82	9.73	11.49	9.91	10.62	11.64	11.92
	Hb(g%)	13.61	12.74	13.52	11.94	12.83	11.66	12.23	12.56	13.14	12.43	13.75	12.87

A: General Condition

B: Hematology examine

Q: Normal

桂林医学院实验记录

Experiment Record of Guilin Medical College

罗汉果提取物 3g/kg/天灌服 90 天后对实验狗 (1) 五种器官病理学检查结果

Five organ's pathology examination results of the dog (1) with extract of Luohanguo 3g/kg/day i.g. for 90days.

No. 1

器官 Organs	重量(g) Weight	外观 Appearance		浊肿 Turbid swelling	空泡变性 Vacuolar degeneration	炎症灶 Inflammatory focus	坏死灶 Necrotic focus
肝 Liver	368.4	无异常 Without	Abnormal	无 None	无 None	无 None	无 None
心 Heart	97.32	无异常 〃	〃	无 None	无 None	无 〃	无 None
肾 kidney	38.60	无异常 〃	〃	无 〃	无 〃	无 〃	无 〃
肺 Lung	218.20	无异常 〃	〃	无 〃	无 〃	无 〃	无 〃
脾 Spleen	60.41	无异常 〃	〃	无 〃	无 〃	无 〃	无 〃

(1)号狗试验记录表:Experiment Record Table of dog (1).

	指标 Index	日期(天) Date											
		0	7	14	21	28	35	42	49	56	63	77	90
血液生化检验F	血钾(mmol/L) Blood K^+	4.30	4.27	4.08	4.11	4.32	4.05	4.07	4.12	4.20	4.25	4.30	4.16
	血磷(mmol/L) Blood P^{5+}	1.76	1.74	1.82	1.69	1.77	1.75	1.81	1.84	1.67	1.69	1.57	1.49
	血氯(mmol/L Blood Cl^-	98.60	100.00	97.50	99.28	98.10	92.20	112.30	115.40	120.50	132.10	118.10	120.20
	血钙(mmol/L) Blood Ca^{2+}	1.70	1.82	1.90	2.00	2.12	2.30	1.92	1.73	1.80	1.93	1.76	1.94

F: Blood biochemistry examine

桂林医学院实验记录

Experiment Record of Guilin Medical College

（1）号狗试验记录表：（接上表）Experiment Record Table of dog (1)

No. 1

	指标 Index	日期（天）Date											
		0	7	14	21	28	35	42	49	56	63	77	90
肝功能检验C	TP(g/L)	60.14	58.26	52.35	59.26	61.74	58.34	58.69	60.53	58.45	61.26	56.22	59.
	ALB(g/L)	26.58	27.12	26.46	25.66	24.78	28.45	26.24	25.67	26.12	27.24	24.59	26.
	GLO(g/L)	28.54	31.46	26.43	25.26	32.14	26.67	28.46	30.48	29.14	28.26	31.42	32.
	ALT(u/L)	31.44	28.67	30.25	28.58	29.64	31.27	30.54	28.44	31.25	28.41	28.48	31.
	AST(u/L)	26.41	30.12	28.42	31.23	26.91	28.42	32.43	26.96	30.24	28.68	27.61	29.
肾功能检验D	尿量（ml/日）Urine Volume(ml/day)	310.25	280.13	290.41	260.82	272.31	281.24	290.53	310.57	323.86	294.31	261.45	310.
	尿液 PH Urine PH	6.2	6.1	6.0	5.9	5.8	6.2	6.0	6.5	6.4	6.0	5.9	6.1
	BUN(mmol/L)	3.24	3.67	4.15	4.27	3.45	4.64	3.81	4.23	4.11	3.92	4.26	3.84
	Cr(mmol/L)	88.27	76.45	72.37	62.54	71.15	61.59	72.41	75.63	66.41	76.13	62.45	70.
	红蛋白（g/L）Red Protein	0	0	0	0	0	0	0	0	0	0	0	0
血尿常检验E	血糖（mmol/L）Blood sugar	5.24	5.57	4.33	4.90	5.26	4.85	5.10	4.64	5.21	5.68	5.28	4.87
	尿糖（mmol/L）Urine Sugar	0	0	0	0	0	0	0	0	0	0	0	0

C: Liver function examine.

D: Kidney function examine.

E: Blood sugar and urine sugar examine.

桂林医学院实验记录

8只的平均值

Experiment Data Sheet

蒸水 10ml/kg/日连续灌服 90 天 8 只狗(第 2,3,4,7,10,12,14,16,号狗)各项检查结果统计表

tistic calculations of results of the Examinations of the eight dogs(dogsNo.2,3,4,7,10,12,14,16)of the control group (gavaged with distilled water 10ml/kg/day for 90 days .)

Tab A.B.

指标 Index	动物数 (n)	日期(天) Date											
		0	7	14	21	28	35	42	49	56	63	77	90
体重(kg) Body Weight	8	8.06±0.28	8.18±0.27	8.31±0.30	8.56±0.32	8.69±0.29	8.91±0.39	9.21±0.45	9.36±0.43	9.57±0.44	9.83±0.49	10.11±0.63	10.34±0.64
外观毛色 Hair Appearance	8	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
活动情况 Active(Condition)	8	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
心率(次/分) Heart Rate(time/min)	8	171.38±13.96	172.50±16.48	176.38±14.17	176.50±16.71	175.38±14.42	175.00±8.73	174.25±10.04	173.25±10.10	176.00±10.89	176.50±9.02	177.13±10.01	176.63±11.45
血压(mmHg) Blood Pressure	8	121.38/72.38 ±8.93/±6.55	121.25/74.63 ±5.20/±6.52	120.63/72.00 ±6.76/±6.16	122.75/74.50 ±7.17/±6.28	124.88/75.38 ±6.92/±6.57	121.88/74.25 ±13.38/±8.41	122.00/75.50 ±10.77/±6.21	122.63/75.50 ±9.58/±6.46	123.13/75.38 ±7.83/±6.72	121.00/75.25 ±6.32/±7.89	126.13/75.25 ±7.53/±7.71	127.00/76.25 ±4.66/±7.85
呼吸(次/分) Respiration(time/min)	8	31.88±4.55	28.88±2.95	32.63±5.07	30.00±5.78	30.38±4.82	29.75±3.62	30.50±4.99	31.38±4.63	32.13±2.56	33.50±5.48	33.25±5.37	32.75±4.45
大便 Stool	8	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
小便 Urine	8	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
RBC:×10⁶/mm³	8	5.38±0.31	6.12±0.52	6.16±0.42	6.10±0.43	6.06±4.82	6.36±0.53	6.57±0.41	6.75±0.48	6.77±0.47	6.85±0.43	6.41±0.58	6.81±0.38
WBC:×10³/mm³	8	11.06±1.61	10.52±1.33	10.70±1.49	11.55±0.76	11.00±0.87	11.19±1.22	11.72±0.94	10.58±3.35	11.61±1.21	11.69±1.40	11.24±0.99	11.65±0.87
Hb: g%	8	12.60±0.34	12.33±1.07	12.38±1.03	12.76±0.85	12.68±0.96	12.66±0.87	12.84±1.25	13.34±0.38	12.34±1.35	12.43±1.27	12.57±0.74	12.39±1.09

A: General Condition

B: Hematology examine

Q: Normal

桂林医学院实验记录

Data Sheet

蒸馏水 10ml/kg/日连续灌服 90 天 8 只狗(第 2,3,4,7,10,12,14,16 号狗)各项检查结果统计表

Statistic calculations of results of the Examinations of the eight dogs(dogsNo.2,3,4,7,10,12,14,16)of the control group (gavaged with distilled water 10ml/kg/day for 90 days .)

Fab.F.

指标 Index		动物数 (n)	日期(天) Date											
			0	7	14	21	28	35	42	49	56	63	77	
血液生化检验 F	血钾(mmol/L) Blood K^+	8	4.12±0.10	4.18 ±0.09	4.18 ±0.13	4.25 ±0.15	4.28 ±0.10	4.20 ±0.16	4.24 ±0.19	4.22 ±0.07	4.28 ±0.08	4.24 ±0.14	4.23 ±0.19	4.2 ±
	血磷(mmol/L) Blood P^{-1}	8	1.78 ±0.08	1.72 ±0.13	1.66 ±0.13	1.67 ±0.16	1.76 ±0.15	1.91 ±0.13	1.94 ±0.14	1.99 ±0.19	1.80 ±0.16	1.81 ±0.09	1.75 ±0.09	1.8 ±
	血氯(mmol/L Blood CL^-		113.4 ±10.45	112.9 ±6.01	113.3 ±7.54	113.6 ±13.47	113.2 ±9.22	121.6 ±27.48	112.6 ±28.45	114.0 ±3.40	115.6 ±8.44	113.6 ±5.34	116.0 ±11.38	118. ±
	血钙(mmol/L) Blood Ca^{2+}		1.83 ±0.11	1.71 ±0.13	1.76 ±0.09	1.68 ±0.31	1.80 ±0.36	1.91 ±0.21	1.80 ±0.22	1.89 ±0.07	1.73 ±0.15	1.63 ±0.25	1.80 ±0.21	1.7 ±

F: Blood biochemistry examination

蒸馏水 10ml/kg/日连续灌服 90 天 8 只狗(第 2,3,4,7,10,12,14,16 号狗)各项检查结果统计表

Statistic calculations of results of the Examinations of the eight dogs(dogsNo.2,3,4,7,10,12,14,16)of the control group (gavaged with distilled water 10ml/kg/day for 90 days .)

Fab G.

器官 Organs	动物数 (n)	重量(g) Weight	外观 Appearance	混肿 Turbid swelling	空泡变性 Vacuolar degeneration	炎症灶 Inflammatory focus	坏死灶 Necrotic focus
肝 Liver	8	348.3±14.84	无异常 without abnormal	无 None	无 None	无 None	无 None
心 Heart	8	99.9±1.11	无异常 "	无 None	无 None	无 None	无 None
肾 kidney	8	93.1±2.12	无异常 "	无 None	无 None	无 None	无 None
肺 Lung	8	226.6±7.45	无异常 "	无 None	无 None	无 None	无 None
脾 Spleen	8	63.2±4.99	无异常 "	无 None	无 None	无 None	无 None

G: Organ's pathology examination

桂林医学院实验记录

Experiment Data Sheet

蒸馏水 10ml/kg/日连续灌服 90 天 8 只狗(第 2,3,4,7,10,12,14,16 号狗)各项检查结果统计表

Statistic calculations of results of the Examinations of the eight dogs(dogsNo.2,3,4,7,10,12,14,16)of the control group (gavaged with distilled water 10ml/kg/day for 90 days .)

Tab.C.D.E

	指标 Index	动物数 (n)	日期（天） Date											
			0	7	14	21	28	35	42	49	56	63	77	90
肝功能检验 C	TP（g/L）	8	57.70±6.68	61.71±2.40	54.16±3.83	56.93±4.18	60.05±2.52	60.90±3.69	59.11±4.71	55.40±3.40	62.17±3.62	61.20±3.69	61.76±2.28	60.61±2.66
	ALB(g/L)	8	28.86±0.90	26.73±2.66	26.62±1.60	28.22±1.07	27.00±2.70	28.45±5.12	27.21±1.96	28.80±1.96	28.91±1.39	27.29±1.03	27.82±2.40	28.29±3.45
	GLO(g/L)	8	31.93±1.58	30.92±2.31	30.38±1.86	30.35±4.45	28.51±1.89	27.54±2.95	31.49±6.29	29.76±3.45	29.49±5.31	28.35±1.45	29.01±2.64	29.70±2.22
	ALT(u/L)	8	21.72±1.58	23.31±1.23	24.21±2.97	26.73±3.20	24.85±2.67	27.07±3.70	24.28±2.16	25.46±2.89	26.63±2.94	28.11±3.80	25.64±2.63	26.88±3.52
	AST(u/L)	8	30.28±7.72	35.21±3.33	30.89±3.69	30.12±3.20	26.10±4.06	28.30±3.97	27.15±3.60	27.82±3.32	29.88±5.60	27.43±3.35	27.84±3.51	27.73±2.78
肾功能检验 D	尿量（ml/日） Urine Volume(ml/day)	8	326.36±11.03	340.45±7.58	335.19±59.09	315.71±37.53	297.56±39.64	312.45±48.52	331.33±23.01	355.49±20.43	342.16±32.35	343.65±10.08	370.97±39.83	368.19±16.3
	尿液 PH Urine PH	8	6.31±0.27	6.23±0.14	6.40±0.23	6.25±0.28	6.15±0.40	6.24±0.52	6.08±0.36	6.29±0.38	6.23±0.37	6.10±0.24	6.21±0.36	6.24±0.13
	BUN(mmol/L)	8	3.61±0.46	3.33±0.35	3.56±0.28	3.81±0.37	4.08±0.54	3.69±0.55	4.16±0.48	3.39±1.04	3.76±0.73	3.40±0.59	3.92±0.86	3.77±0.76
	Cr(mmol/L)	8	82.50±11.18	81.21±9.43	73.86±8.11	70.27±4.55	77.39±3.04	71.16±8.45	74.78±4.90	74.91±5.29	79.71±2.40	80.16±7.82	80.03±6.79	80.32±4.15
	红蛋白（g/L） Red Protein	8	0.0±0.0	0	0	0	0	0	0	0	0	0	0	0
血尿糖检验 E	血糖（mmol/L） Blood sugar	8	5.78±0.64	5.34±0.71	5.44±0.47	5.57±0.92	5.22±0.74	5.92±0.87	5.28±0.40	5.14±0.39	5.05±0.37	5.13±0.19	5.28±0.18	5.27±0.53
	尿糖（mmol/L） Urine Sugar	8	0	0	0	0	0	0	0	0	0	0	0	0

C: Liver function examination .

D: Kidney function examination .

E: Blood sugar and urine sugar examination

一只狗的第8份

桂林医学院实验记录

Experiment Record of Guilin Medical College

No. 2

(2)号狗试验记录表: Experiment Record Table of dog (2)

	指标 Index	日期(天) Date											
		0	7	14	21	28	35	42	49	56	63	77	90
一般情况A	体重(kg) Body Weight	8.0	8.2	8.3	8.5	8.6	8.7	8.9	9.1	9.2	9.5	9.6	9.8
	外观毛色 Hair Appearance	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q
	活动情况 Active(Condition)	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q
	心率(次/分) Heart Rate(time/min)	176	182	184	191	185	182	181	178	176	189	190	188
	血压(mmHg) Blood Presure	108/70	121/81	109/69	110/72	120/82	131/86	107/78	113/76	125/78	120/82	125/83	128/84
	呼吸(次/分) Respiration(time/min)	36	29	35	27	38	31	38	35	32	33	39	34
	大便 Stool	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q
	小便 Urine	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q	正常 Q
血液学检验B	RBC: $\times 10^6/mm^3$	5.27	6.26	6.37	5.93	6.15	7.10	7.15	7.20	6.38	7.02	5.97	6.07
	WBC: $\times 10^3/mm^3$	8.28	9.13	10.54	11.28	9.67	8.58	12.01	11.24	12.13	11.47	11.58	12.04
	Hb(g%)	12.82	12.07	13.21	13.71	14.01	13.29	13.55	13.29	12.87	12.01	12.87	12.44

A: General Condition

B: Hematology examine

Q: Normal

桂林医学院实验记录

Experiment Record of Guilin Medical College

罗汉果提取物 3g/kg/天灌服 90 天后对实验狗 ① 五种器官病理学检查结果

Five organ's pathology examination results of the dog ② with extract of Luohanguo 3g/kg/day i.g. for 90days.

No. 2

器官 Organs	重量(g) Weight	外观 Appearance	浊肿 Turbid swelling	空泡变性 Vacuolar degeneration	炎症灶 Inflammatory focus	坏死灶 Necrotic focus
肝 Liver	354.61	无异常 Without Abnormal	无 None	无 None	无 None	无 None
心 Heart	92.73	无异常 Without Abnormal	无 None	无 None	无 None	无 None
肾 kidney	39.25	无异常 Without Abnormal	无 None	无 None	无 None	无 None
肺 Lung	217.52	无异常 Without Abnormal	无 None	无 None	无 None	无 None
脾 Spleen	58.64	无异常 Without Abnormal	无 None	无 None	无 None	无 None

(2)号狗实验记录表:Experiment Record Table of dog (2).

指标 Index		日期(天) Date											
		0	7	14	21	28	35	42	49	56	63	77	90
血液生化检验 F	血钾(mmol/L) Blood K^+	4.32	4.16	4.07	4.13	4.25	4.26	4.06	4.11	4.50	4.32	4.18	4.31
	血磷(mmol/L) Blood P^{-3}	1.69	1.82	1.84	1.77	1.92	2.10	1.83	1.81	1.69	1.75	1.76	1.83
	血氯(mmol/L Blood Cl^-	102.3	110.4	116.5	98.2	120.6	111.3	97.8	120.4	123.6	115.7	120.8	121.4
	血钙(mmol/L) Blood Ca^{2+}	1.81	1.92	1.78	1.69	2.12	2.31	1.23	1.67	1.82	1.77	1.80	1.69

F: Blood biochemistry examine

桂林医学院实验记录

Experiment Record of Guilin Medical College

(2) 号狗试验记录表：（接上表）Experiment Record Table of dog (2)

No.2

	指标 Index	日期（天）Date											
		0	7	14	21	28	35	42	49	56	63	77	90
肝功能检验C	TP（g/L）	56.42	61.43	59.47	51.28	60.11	62.34	58.84	56.22	61.01	58.20	62.01	56.(
	ALB(g/L)	28.12	24.62	27.01	28.04	26.45	25.00	27.12	28.46	27.77	26.40	25.15	24.
	GLO(g/L)	31.24	30.43	28.25	27.40	26.28	22.80	24.53	26.25	21.10	28.62	27.41	28.
	ALT(u/L)	22.33	24.50	20.17	23.54	26.12	25.20	24.64	23.26	26.10	27.21	26.60	25
	AST(u/L)	37.17	38.22	28.87	29.63	20.88	26.28	24.92	25.12	26.72	27.14	25.43	24
肾功能检验D	尿量（ml/日）Urine Volume(ml/day)	320.14	330.20	350.50	354.10	280.20	290.10	360.34	389.24	390.50	350.24	385.10	37
	尿液 PH Urine PH	6.3	6.1	6.5	6.7	6.8	5.8	5.7	6.8	6.7	6.6	6.5	6.
	BUN(mmol/L)	3.30	3.25	3.22	3.47	4.89	4.21	4.18	3.28	3.67	4.66	4.29	4.8
	Cr(mmol/L)	88.07	79.28	62.14	72.58	82.24	84.12	81.24	80.22	82.01	84.51	82.61	8.
	红蛋白（g/L）Red Protein	0	0	0	0	0	0	0	0	0	0	0	
血尿糖检验E	血糖（mmol/L）Blood sugar	5.64	4.42	5.28	4.92	4.58	5.34	4.82	5.33	4.92	5.21	5.47	4.
	尿糖（mmol/L）Urine Sugar	0	0	0	0	0	0	0	0	0	0	0	

C: Liver function examine .

D: Kidney function examine .

E: Blood sugar and urine sugar examine.

THE SEARCH FOR NONCARIOGENIC SWEETENING AGENTS FROM PLANTS

A. Douglas Kinghorn [1)]

ABSTRACT

Higher plants are known to biosynthesize many structurally diverse secondary metabolites that are intensely sweet. Several plant-derived compounds already have found commercial application as noncariogenic and noncaloric sucrose substitutes for sweetening foods, beverages, and medicines. Therefore, plants with highly sweet constituents are worthy of consideration as high-yielding crop plants for the production of sweet constituent per se, can be cultivated to produce compounds that can be chemically modified to generate sweet substances. In this paper, four types of naturally occuring intense sweeteners will be considered that are in different stages of current development, namely, hernandulcin, a newly discovered sweet sesquiterpene; two classes of triterpene glycosides, periandrins I-IV and mogroside V, whose structures were reported relatively recently; and stevioside, the major sweet diterpene glycoside constituent of the Paraguayan herb, Stevia rebaudiana, which is presently used as an approved sweetener in Japan. In the case of the latter substance, it has recently been found that steviol, the enzymatically produced aglycone of stevioside, is mutagenic in a metabolically active form, when tested in a forward mutation assay using Salmonella typhimurium strain TM677. Progress made in the identification of mutagenic steviol synthetic analogs and in vitro metabolites will be reviewed. Finnally, speculation will be made on the future directions of research on naturally occuring sweetening agents.

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INTRODUCTION

Sucrose, the most abundant free sugar in plants, is increasing in consumption on a world-wide basis both as a nutritional agent and as a sweetener (1,2). It Has been estimated that about 80% of the annual increase in the use of sucrose is occuring in developing countries (2). Sucrose, the most widely used sweetener, has several properties in addition to its taste attributes that make it useful as a food additive, such as providing bulk, acting as a preservative, and producing color and flavor when thermally degraded or caramelized (2).

Rather large amounts of sucrose, however, are used to sweeten foods, which normally contain concentration levels equivalent to between 5 and 12% w/v. As a consequence of the widespread use of sucrose in foods, beverages, and medicines, it is now recognized that this substance is the primary cause of dental caries in the western diet (3). Furthermore, an alarming increase in dental caries is now apparent in developing countries, which has been attributed to a greatly increased consumption of sucrose and other refined sugars in soft drinks, foods, and confections (4).

Since it has been found that restricting the dietary intake of sucrose can limit the number of cariogenic colonies of Streptococcus mutans, the main pathogen in caries for man (5), a number of noncariogenic sweetening agnets have been introduced as sucrose substitutes. The demand for these substances is great because several alternative sweeteners are also regarded as being noncaloric. However, the criteria that must be satisfied prior to the introduction of a new sweetening agent into the market are exacting, and include, in addition to sweetness, the need for such a substance to have a pleasant taste similar to that of sucrose, and to produce no toxic or cariogenic effects, either in the unmetabolized or metabolized form (6). A sweet substance should also be odorless and colorless, and exhibit water-solubility and chemical and thermal stability, and should be easy to synthesize or to obtain after cultivation, as well as being capable of fitting into existing techniques for the application of sweeteners (6). A final prerequisite is that a sweet compound should be economically competitive with existing sweeteners (6). No one sweetener conforms with all of these diverse criteria, and, for example, a number of concerns have arisen relative to the safety of the three most extensively used low-calorie sweeteners. Thus, saccharin, which is used in the United States and in more than 80 other countries throughout the world, has been demonstrated as a bladder carcinogen for female Swiss mice (7) and male Sprague Dawley rats (8), and may also be an urinary bladder tumor promoter (9). Aspartame, which is now approved in the U.S. and in 50 other countries, is contra-indicated for persons suffering from phenylketonuria, and reservations have been expressed that high plasma levels of

aspartate, occuring as a result of the ingestion of this sweetener, may lead to undersirable effects on neuroendocrine regulating systems, and to mental retardation and brain damage (10). In addition, clinical case study data have been described that suggest that the consumption of very high levels of aspartame may be related to the incidence of seizures (11). Finally, cyclamate, which is available as a sweetener in about 40 countries, although not currently in the United States, has been found to metabolize to cyclohexylamine, which is known to cause testicular atrophy in rats (12). In addition, cyclamate has been associated with the development of bladder tumors in rats fed a 10:1 mixture with saccharin (12), and there is some evidence that cyclamate may act as a bladder tumor promoter *in vivo* (13).

The search for noncariogenic sweetening agents from plants has been greatly stimulated by perceived problems and limitations of use of existing sucrose substitutes. The types of sweet compounds produced by plants can be broadly classified into the sugar (bulk, nutritive) and non-sugar (hihg-intensity, low-caloric) types. While the first category has been known since prehistoric times, representatives of the latter group, which are often hundreds or even thousands of times sweeter than sucrose, have only become known relatively recently. It is the latter group of compounds that will be focussed on in this chapter, and such compounds, if they are not carbohydrates and are used in low concentrations, can be regarded as being noncariogenic (14). A classification of the various structural types of natural intensely sweet compounds is provided in Table 1.

It may be pointed out that a number of sweet plant-derived compounds are already used commercially as sweetening, flavoring, or taste-modifying agents, and these comprise glycyrrhizin (from *Glycyrrhiza glabra* L.; Leguminosae), thaumatin [from *Thaumatococcus daniellii* (Bennett) Benth.; Marantaceae]; stevioside [from *Stevia rebaudiana* (Bertoni) Bertoni; Compositae], and phyllodulcin [from *Hydrangea macrophylla* (Thunb.) Seringer var. *thunbergii* (Siebold) Makino] (15-20). In addition, chemically modified derivatives of plant constitutents also may have use in this regard as additives for foods, beverages, medicines, and tobacco, such as perillartine the [α -syn-oxime of perilladehyde, a compound obtained from the essential oil of *Perilla frutescens* (L.) Britton], neohesperidin dihydrochalcone (obtained from neohesperidin in the peels of the Seville orange, *Citrus aurantium* L.), and ammoniated glycyrhizin (the fully ammoniated salt of glycyrrihizic acid obtained from licorice, *G. glabra*) (15-18, 20-21). Under the right circumstances, therefore, plants that yield intensely sweet extracts/or compounds can be considered for commercial cultivations as crop plants.

The remainder of this paper will be devoted mainly to reviewing progress made on four categories of intensely sweet plant constituents, that have been studied in the laboratories of the College of Pharmacy, University of Illinois at Chicago. These compounds are all terpenoids, but are in different stages of current development. The

first compound to be reviewed will be hernandulcin, an intensely sweet sesquiterpene alcohol that was recently discovered at this institution (22). Two types of triterpene glycosides will then be discussed that, after their initial isolation and characterization in Japanese laboratories a few years ago, have only received preliminary evaluation as to their suitability for use as sweetening agents, namely, periandrins I-IV (23-25) and mogroside V (26-28). Finnaly, attention will be turned to stevioside, a diterpene glycoside mentioned previously that was first discovered some eighty years ago (29), but whose structure was not determined until about sxty years later (30,31). Extracts of its plant of origin, Stevia rebaudiana, and stevioside itself are currently not approved as sucrose substitutes in the United States, but are used in Japan to sweeten Japanese-style pickles, dried seafoods, fish meat products, soy sauce containing products, confectionery products, soft drinks, and chewing gum (18,19). S. rebaudiana ectracts are available for sale in Paraguay to sweeten mate and are prescribed by physicians for the treatment of hyperglycemia (32). However, despite this reasonably wide use of stevioside and S. rebaudiana products by human populations without apparent harmful effects, we have found that the stevioside aglycone, steviol, is mutagenic under certain conditions that will be described in this review.

DISCOVERY OF THE SWEET SESQUITERPENE, HERNANDULCIN

Hernandulcin was discovered at this institution during the course of the graduate studies of Dr. Cesar M. Compadre. Dr. Comprade, a native of Mexico, was familiar with texts describing the use of medicinal plants by the Aztecs, and in one such book, entitled Natural History of New Spain, written between 1570 and 1576, and published in Latin in 1651, the Spanish physician, Francisco Hernandez, referred to a remarkably sweet plant under the Nahuatl name Tzonpelic xihuitl (22,34). The work of Hernandez has been translated several times into Spanish, and in the latest Spanish edition (35), the plant is listed under the name Tzopelicxihuitl. In the Nahuatl language, the noun xihuitl or xiuitl means herb, and the adjective tzopelic means sweet, and therefore the name of the plant can be translated literally as meaning "sweet herb" (35). A translation of part of the description in Spanish given by Hernandez (35) for this sweet plant is as follows (36):

"The leaves of this herb are sweet to such a degree that honey, sugar and all the other substances used to sweeten appear to be inferior, as if, in this plant, nature were trying to determine how much sweetness it could impart to natural things".

While conducting field work in Mexico in 1981 and 1982, it was discovered by members of our group that the same plant described and illustrated by Hernandez was still available for sale in marketplaces under the name, hierba dulce. This plant is apparently not used for sweetening foods, beverages or medicines, but is now employed for the treatment of coughs and for a reputed abortifacient activity (36). Hierba dulce, and by inference, Tzonpelic xihuiti, were identified as Lippia dulcis Trev. (Verbenaceae), and an intensely sweet bisabolane sesquiterpene, which was named hernandulcin in honor of Hernandez, was isolated from the leaves and flowers of the plant after solvent partition and chromatographic fractionation. The structure of hernandulcin (Fig. I,1) was established as 6-(1,5-dimethyl-1-hydroxyhex-4-enyl)-3-methylcyclohex-2-enone as a result of the application of spectroscope techniques inclusive of high-resolution mass spectrometry and two-dimensional NMR, and was confirmed by its synthesis, which involved a directed aldol condensation (22).

Synthetic (±)-hernandulcin did not induce bacterial mutation when examined in a forward mutation assay utilizing Salmonella typhimurium strain TM677, and was nontoxic for male Swiss-Webster mice at single doses up to 2 g/kg body weight, when administered by oral intubation. A human taste panel rated the naturally 0.25 M sucrose solution on a molar basis, although it was perceived to be somewhat less pleasant than sucrose, and to exhibit some bitterness and aftertaste (22).

At this stage, it does not seem that it will be feasible to produce hernandulcin from cultivated L. dulcis plants, since the compound occurs in very low yield (0.004% w/w) in the dried herb (36). It is unlikely also that partially purified L. dulcis extracts could be used for sweetening purposes, because such extracts would be expected to be contaminated with camphor, a compound which co-purified with hernandulcin. Camphor, which is regarded as a very toxic compound, was found to constitute as much as 53% w/w of the sweet volatile oil of L. dulcis after analysis by gas chromatography/mass spectrometry (36). The symptoms of camphor poisoning are most common in small children, and include nausea, vomiting, CNS depression, and coma (37). Camphor crosses the placenta, and has been associated with neonatal death (38-40), and therefore, it is possible that its high concentration in L. dulcis herb is the basis for the belief in Mexico that this plant has an abortifacient action (36).

Future evaluation of hernandulcin will thus be more easily be performed on the synthetic racemic form rather than on the naturally occuring (+)-enantiomer. Hernandulcin still requires detailed laboratory study to determine its suitability for application as a sweetening agent, especially in regard to its stability, solubility, safety, and potential utility. The discovery of hernandulcin underscores the value of plant constituents as intensely sweet molecules, and this compound is the prototype of a new class of intense sweeteners, that will no doubt be modified synthetically in the attempt to produce a compound or compounds with improved sweetness parameters. It is interesing to note that two postulated binding sites with the sweetness receptor in hernandulcin, namely, the C-1 carbonyl and the C-1' hydroxy group, are located about 2.6 Å apart, and are in the preferred conformation to closely fit the Shallenberger model (41) for sweet-tasting compounds (22).

ASSESSMENT OF PERIANDRINS I-IV AND MOGROSIDE V AS SWEETENERS

1. Periandrins I-IV

In 1941, the sweet taste of the roots of Periandra dulcis Mart. (leguminosae) (brazilian licorice) was attributed to the presence of 0.38% w/w glycyrrhizin (42). More recent studies by Hashimoto and colleagues (23-25, 43), however, have shown that the sweet principles of this plant are four oleanane-type triterpene glycosides, namely, periandrins I, II, III and IV (Fig. 1, 2,3,4,5, respectively). The structure of periandrins I was confirmed by X-ray crystallography (25). These compounds were isolated in pure form in rather low yield, and 700 mg, 170 mg, 23 mg, and 9 mg of periandrins I-IV, respectively, were obtained from 30 kg of P. dulcis dried roots (43).

We have found that these four compounds were not mutagenic in a forward mutation assay utilizing Salmonella typhimurium strain TM677, either in the presence or absence of a mutagenic activator (44). In addition, one of the compounds, periandrin II, exhibited no toxicity for mice at up to 2 g/kg body weight, in an acute toxicity experiment (44). Periandrins I-IV have each been found to exhibit approximately 90 times the sweetness intensity of sucrose, as shown in Table 1. These P. dulcis constituents are claimed to

have a considerably less bitter taste than glycyrrhizin, and to have a more rapid onset of sweetness (43). We have found periandrins I and II to be extremely stable when stored in solution at pH 2 through 12 for over two months at both room temperature and 60° C. In addition, no measurable breakdown of these compounds was observed when heated in the dry state at 100° C for 24 hours (44).

While the periandrins are interesting in serving as yet another structural class of plant-derived sweetening agents, the comparative difficulty involved in their isolation and the low individual yields of these compounds will probably serve to prohibit their widespread use as sucrose substitutes. In addition, aqueous or alcoholic P. dulcis root extracts would not be useful as sweeteners or sweetener aids, beacuse they contain uncharacterized bitter components in addition to the sweet periandrins I-IV (43).

2. Mogroside V

Thladiantha grosvenorii (Swingle) C. Jeffrey (formerly Momordica grosvenori Swingle (Curcurbitaceae) is a vine which is cultivated by the Miao-tze people of Kwangsi Province in the People's Republic of China (45,46). This is an intensely sweet-tasting fruit by the name of Lo Han Kuo, which has been sold in south China as a remedy for colds, sore throats, and minor stomach and intestinal troubles (45). According to Swingle, about 1.000 tons of the green fruits of Lo Han Kuo were delivered to drying sheds at Kweilin, to be shipped to Canton for domestic consumption in Chine and for export to the United States (45). One proprietary product we purchased in the United States "Lo-Han-Kuo Infusion" (Lo Han Kuo Products Manufactory, Kwangsi, People's Republic of China) is recommended for use as an antipyretic, and for enriching the lungs, cough relief, and dissipating sputum. It is formulated with 95% Lo Han Kuo (Fructus Momordicae) and 5% cane sugar (presumably added to assist in the formulation, rather than for sweetening), and is suggeted to be taken by dissolving one piece of tableted infusion (about 10 g) by dissolution into 100 ml of boiling water, two or three times a day.

It was established by Lee that a major sweet constituent of Lo Han Kuo fruits is a triterpene glycoside (47). Takemoto and co-workers have described the isolation of three intensely sweet constituents of T. grosvenorii fruits, mogrosides IV-VI, and the structure determination of the most abundant of these, mogroside V (Fig. I, 6) has been published (26-28). These compounds are glycosides based on the aglycone, mogrol (10 - cucurbit-5-en-3 , 11 , 24 (R), 25-tetraol. In our laboratory, we have found that the concentrations of mogroside V in an authenticated T. grosvenorii fruit sample were as follows : peel, 1.26% (as determined by high-performance liquid chromatography)(48).

In other work performed in our laboratory on mogroside V, it has been established that this compound is non mutagenic and produces no mortality in acute toxicity experiments on mice at doses up to 2 g/kg body weight, and it exhibits an equivalent molar sweetness to stevioside when tested againts a standard sucrose solution by a human taste panel (48). At a concentration of 20 mg/100 ml, mogroside V, mogroside VI, and stevioside were rated at 256, 125, and 233 times sweeter than sucrose by a human taste panel in Takemoto's laboratory (28). However, Lee (47) has reported that the intense sweetness of a T. grosvenorii constituent, which was most likely mogroside V, was accompanied by a lingering, licorice-like aftertaste.

It may therefore be concluded that mogroside V, in being intensely sweet, nonmutagenic and nontoxic in preliminary tests, and occuring in the plant in relatively high concentrations, does hold promise as a potential sucrose substitute. In addition, we have determined at our institution that the compound is stable in solution at all pH levels, and is highly water-soluble (44). However, more detailed analyses of its hedonic taste characteristics and its pharmacological activities are clearly necessary. In addition, extracts of T. grosvenorii fruits might be able to be used for sweetening purposes, since they have history of consumption by human populations without apparent harmful effects, and no toxic or bitter constituents of the fruits have been reported. T. grosvenorii would seem to be an atractive species for introduction into other countries from its native China for further investigation of its highly sweet properties.

FURTHER STUDIES ON STEVIOSIDE AND ITS AGLYCONE STEVIOL

1. Overview of the Use of Stevioside and Extracts of Stevia rebaudiana as Sweetening Agents.

The most widespread usage of stevioside and purified Stevia rebaudiana extracts is currently taking place in Japan. Not only is S. rebaudiana grown in Japan itself, but substantial amounts are cultivated in South Korea and Paraguay for the Japanese market (19). The cultivation of the plant has also been reported to occur in Taiwan, the People's Republic of China, Indonesia, Thailand, Laos, and Brazil (19, 49, 50). Recent estimates of the total amount of stevioside consumed per annum in Japan range from about 700 to 1,000 metric tons, with about two thirds of this amount being produced in Japan (19). According to Akashi (51), stevioside is appropriate for use as a sweetening agent

in all foods, since it is highly stable in heat and acids; its sweetness characteristics are similar to those of sucrose; it is mild and free of aftertaste; it can be considered a noncaloric substance; it is nonfermentive; and it does not become yellowish when heated. It has also been shown that stevioside markedly suppresses the growth of certain organisms that cause dental caries, namely, Streptococcus mutans, Lactobacillus plantarum, and L. casei (52). As indicated earlier in this chapter, stevioside is used in a wide variety of food items in Japan, but it does not normally seem to be used at concentration levels greater than 0.1% w/w or w/v (19). Stevioside is suitable for use as sweetening agent in soft drinks, because, unlike glycyrrhizin, it does not precipitate in acid cinditions at around pH 3 (19).

Following the successful structure determination of stevioside (Fig. II, 7) at the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland, U.S.A. (30, 31), a further seven sweet ent-kaurene glycosides were isolated from S. rebaudiana leaves by Japanese groups in the 1970's, namely, steviobioside (Fig. II,8), rebaudioside A (Fig. II,9), rebaudioside B (Fig. II,10), rebaudioside C (Fig. II, 11), rebaudioside D (Fig. II,12), rebaudioside E (Fig. II, 13) and dulcoside a (Fig. II, 14) (53-56). These compounds occur in remarkably high concentrations in S. rebaudiana leaves, and in a dried sample obtained from the People's Republic of China, we have recently shown that the yields of stevioside, rebaudioside A, rebaudioside C, and dulcoside A were, respectively, 6.6, 3.7, 2.1, and 0.53% w/w (50). Such compounds, which are ent-kaurene glycosides, are apparently very rare in the genus Stevia, despite occuring in such abundance in S.rebaudiana. When 110 Stevia leaf herbarius specimens were examined phytochemically, stevioside was detected in only two spesies, S. rebaudiana, collected in Paraguay in 1919, and S. phlebophylla A. Gray, collected in Mexico, in 1889. Rebaudiosides A and C were also identified in the stored S. rebaudiana sample, and both specimens were still perceptibly sweet when we examined them (57).

Stevioside, when tasted in almost pure form, is characterized by a prolonged aftertaste and exhibits considerable bitterness and astringency (58). This compound has been rated about 300 times sweeter than a 0.4% w/w sucrose solution, and 150-fold sweeter than 10% w/w sucrose (59). According to Tanaka (59), rebaudioside A, with a branched sugar chain unit affixed to C-13, is some 30% sweeter than stevioside. The replacement of a sucrose unit of rebaudioside A eith a rhamnose substituent, as in rebaudioside C, produces a considerably less sweet and less pleasant-tasting molecule (55,59). Considerable effort has been expended producing semisynthetic analogues of stevioside that are not bitter, and DuBois and Stephenson (60) have reported some success in this endeavor.

There is extensive literature on S. rebaudiana extracts and stevioside as sweetening agents, much of which has emanated from Japan. Over 100 Japanese patented procedures have covered aspects of the extraction, purification, and taste improvement of this sweet glycoside (16,18-20).

2. Safety Studies on Stevioside and Steviol

While Stevia rebaudiana extracts and purified stevioside are used as high-intensity sweeteners in Japan and in certain other countries, firm evidence of their safety for human consumption will have to be presented to the Food and Drug Administration before their authorization for use in the United States for this purpose. However, these does exist a body of literature concerning the toxicity of stevioside and S. rebaudiana extracts, that will be briefly reviewed in the following paragraphs.

Crude and refined S. rebaudiana extracts, as well as crystallized stevioside, have been tested for acute and subacute toxicity in mice or rats by several groups, and lethalities were recorded only at very high doses (19, 61, 62). Thus, it was found that aqueous extracts of S. rebaudiana leaves, containing up to 7.0% w/w of stevioside, when mixed with laboratory chow and fed to male and female rats for up to 3 months, produced no remarkable toxic effects (61). In another study conducted with water extracts of the plant containing the equivalent of 0.25 g and 0.5 g stevioside that were incorporated into the diet of rats for 56 days, there appeared no abnormalities relative to controls, other than a significant decrease in serum LDH levels (62). The feeding of S. rebaudiana extracts containing known amounts of stevioside to female rats led to the observation of no skeletal or other teratogenic effects (63).

A number of publications have reported the activity of S. rebaudiana extracts and stevioside in other physiological systems. While it has been claimed there is an experimental basis for the use of the leaves and stems of the plant (in the form of a tea) as a contraceptive agent by the Matto Grasso Indians in Paraguay (64), workers in several other laboratories have been to show no effects on mating performance or fertility in lavoratory animals (19, 61,63). Also, no unequivocal evidence has been presented as to the possible blood-pressure lowering effect in humans after drinking teas containing S. rebaudiana (19). In addition, there is some divergence of opinion as to whether the practice of treating hyperglycemia with S.rebaudiana extracts has any scientific rationale. For example, investigations by Akashi and Yokoyama (61) and Lee et al. (62) reported no dose-related effects on blood glucose levels when S. rebaudiana extracts were fed to rats for several weeks. However, Suzuki et al. (65) demonstrated a

reduction in liver glycogen and in blood glucose levels in rats fed a high carbohydrate diet containing 10% dried S. rebaudiana leaves. A significant decrease of hyperglycemia has recently been observed by a group from Institut Teknologi Bandung, when a spray-dried S. rebaudiana extract was fed to responsible for such effects, so that this observation could be taken into account in the overall evaluation of this substance as a sweetening agent.

Stevioside and extracts of S. rebaudiana have been extensively tested for mutagenicity, and were inactive in this regard when tested in several laboratories againts strains of Salmonella typhimurium, Escherichia coli and Bacillus subtilis, both in the presence and absence of liver-enzyme metabolic activating systems (19,61,67). Stevioside was also considered inactive as a mutagen when tested againts the Muller-5 strain of Drosophila melanogaster (68). We have found that stevioside was inactive in aforward mutation assay utilizing Salmonella typhimurium strain TM677, both in the absence and presence of a metabolic activating system derived from a 9,000 x g supernatant fraction from the liver of Aroclor 1254-pretreated rats, as were the other S. rebaudiana sweet constituent tested, steviolbioside, rebaudiosides A-E, and dulcoside a (33, 44). However, steviol (Fig. III,15) whether produced from stevioside enzymatically by incubation with pectinase or chemically by treatment with sodium periodate and potassium hydroxide, was highly mutagenic in this system (33). The mutagenicity of steviol (ent-13-hydrxykaur-16-en-19-oic acid) was dependent upon the presence of a metabolic activator and on the addition of NADPH, thereby strongly suggesting that the metabolic activation of steviol to a mutagenic compound or compounds is mediated by cytochrome P-450 (33).

In order to investigate the structural requirements for this observed mutagenic effect of steviol, a number of its derivatives were tested in the same bioassay system. Isosteviol (Fig. III, 16) a compound produced from stevioside by treatment with mineral acids (19) that can also be obtained from steviol by a Meerwein rearrangement (31), was found to exhibit no mutagenic activity, either in the presence or absence of a metabolic activator (34). Similar negative results were obtained for the isomeric compounds, dihydrosteviol A (Fig. III, 17) and dihydrosteviol B (Fig. III, 18), which were produced from steviol by catalytic hydrogenation, and for ent-kaurenoic acid (Fig. III, 19), a compound in which the C-13 hydroxy group of steviol is absent (34). In a later study, 19-O- D- glucopyranosylsteviol (Fig. III, 20), proved to be mutagenic toward S. typhimurium strain TM677 in the presence of the metabolic activating system, although it had a somewhat reduced level of activity when compared with steviol. This monoglucoside of steviol may be regarded as a potential in vivo metabolite of stevioside, and its observed mutagenicity incates the apparent non-involvement of the C-19 carboxylic acid group of steviol in mediating its mutagenic response (69). from the evidence obtained to date, it

may be inferred that the presence of the C-16, C-17 exomethylene group and the C-13 tertiary hydroxy group are necessary for the exhibition of mutagenicity of steviol in the bioassay system we have used (34). The partially purified enzyme, epoxide hydrolase, did not inhibit steviol-induced mutagenicity when incorporated into the assay mixture, suggesting that the active steviol metabolite (or metabolites) is not an epoxide (69).

We have performed some in vitro enzymatic metabolism studies on stveiol, in order to learn more about the structural aspects of its mutagenic metabolite or metabolites. Steviol was incorporated with NADPH and the 9,000 x g supernatant fraction from Aroclor-1254 pretreated rats, and the resultant metabolites were analyzed by capillary gas chromatography/mass spectrometry. Under these conditions, steviol remained mainly unmetabolized, and the major pathway of metabolism proved to be oxidation allylic to the exomethylene group at C-16 (70). While none of the in vitro enzymatic metabolites of steviol identified so far have propen to be mutagenic, 15-oxosteviol (Fig. III,21), a compound not yet detected in vitro metabolism studies, but which could feasibility be produced from steviol by allylic oxidation, was synthesized and found to demonstrate activity as a direct-acting mutagen in the bioassay system used (70). This compound, which did not require metabolic activation in order to elicit its mutagenic effect, may turn out to be only one of several mutagenic analogues of steviol, and further studies to isolate and identify additional in vitro metabolites are necessary.

The generation of a mutagenic compound such as 15-oxosteviolk during the in vivo metabolism of stevioside, would obviously be detrimental to the further development of this diterpene glycoside as a sweetening agent. However, little is known about the metabolism of the S.rebaudiana sweet glycosides to date. Wingard and colleagues (71) showed that borh stevioside and rebaudioside A are degraded to steviol when incubated for several days with rat cecal microflora in vitro. In addition, steviol is completely absorbed from the lower bowel of the rat after oral or intracecal administration (71). These observations, coupled with the results of our mutagenicity studies on steviol, suggest that it would be prudent to study in more detail the toxicity of steviol, which has been known for some time to produce an inhibitory effect on oxidative phosphorylation and to restrain the mitochondrial translocation of adenine nucleotides (72). Therefore, while it may be pointed out that no adverse reactions have been reported to occur as a result of the ingestion of S.rebaudiana extracts and stevioside by human population in Japan in over 10 years of their use, there is a clear need for the determination od the in vivo metabolism, the chronic toxicity, and carcinogenicity of these sweet substances (12). In view of the present uncertainty as to the safety of stevioside and its sweet diterpene analogs for human consuption, further capital investment for the cultivation of S. rebaudiana crops would seem to be inadvisable at this time.

FUTURE RESEARCH DIRECTIONS

It seems likely that there will be a continue need for new noncariogenic and noncaloric sucrose substitutes in the future, and many natural product intensely sweet substances, even with less that ideal properties, could find use in speciality markets (16). Plant-derived sweeteners that prove to be inappropriate for commercial exploitation directly, will serve to provide important new leads for subsequent synthetic modification. Since it is still not possible to design sweet compounds in the laboratory that are not structurally related to existing sweeteners (73), newly discovered sweet molecules from plant sources will add greatly to knowledge on the relationship between sweetness and chemical structure (15).

Plant-derived intensely sweet substances are not only of diverse molecular structure, but are of apparent random distribution among the Angiosperms (20). Future success in the discovery and development of such compounds will require a multidisciplinary effort. In order to find additional uninvestigated sweet plants, systematic searches of botanical and ethnobotanical literature, as well as herbarium notations, should prove useful. Field studies, which focus on interviews with local populations as to the use of plants reputed to be sweet in their community, will assume increasing improtance (20). Phytochemical studies directed toward the isolation of sweet compounds, will require preliminary toxicological evaluation of extracts and fractions to be assessed for sweetness, in order that the toxic risk to human participants can be minimized. However, it may be possible to use experimental animals at this stage, and thus dispense with the need to have human subjects taste plant fractions for sweetness. For example, electrophysiological stimulation of the Mongolian gerbil's gustatory receptors (74), and conditioned taste aversion experiments on the same species (75), might be appropriate when combined for monitoring the sweetness of chromatographic fractions obtained from plant extracts. The structures determination and structural modification of sweet isolates will continue to be facilitated by computer-aided technology. Finally, once new noncariogenic and/or noncaloric intensely sweet compounds are deemed to have commercial value, it can be expected that biotechnology will play an important role in their production either by high-yielding plant cell culture methods, as has been reported for stevioside and rebaudioside a (76), or if the compound is proteinaceous, by recombinant DNA techniques (18,77).

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Table 1.

Relative Sweetness to Sucrose of Various Plant-Derived Intense Sweetening Agents (a,b)

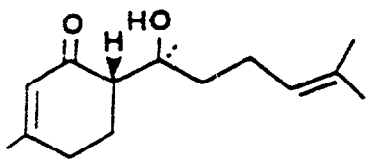
Compound Class	Compound Name	Approximate Sweetness (c) Relative to Sucrose
Monoterpenoid	Perillartine (d)	2000
Sesquiterpenoid	Hernandulcin	1500
Diterpene Glycosides	Stevioside	150
	Rebaudioside A	240
	Rebaudioside D	220
	Rebaudioside E	175
	Rubusoside	115
	Baiyunoside	500
Triterpene Glycosides	Glycyrrhizin	50
	Ammonium glycyrrhizin (d)	50
	Periandrin I	90
	Periandrin II	95
	Periandrin III	92
	Periandrin IV	85
	Mogroside V	340
	Mogroside VI	125
Steroidal Saponin	Osladin	300
Dihydroisocoumarin	Phyllodulcin	400
Dihydrochalcones	Naringin dihydrochalcone (d)	300
	Neohesperidin dihydrochalcone (d)	2000
Proteins	Thaumatin (Talin R protein)	3000
	Monellin	3000

(a) Relative intensity data taken from reference 15.

(b) It should be noted that not all of these quantitative data have been obtained using the same sensory techniques, and that sweetness intensity may vary with several factors including sweetener concentration.

(c) Sucrose = 1.0 ; figures are expressed on a weight comparison basis.

(d) Derivate of natural product.



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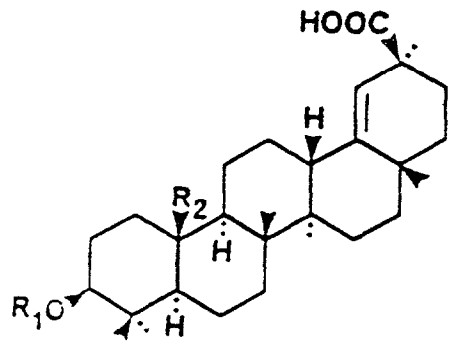
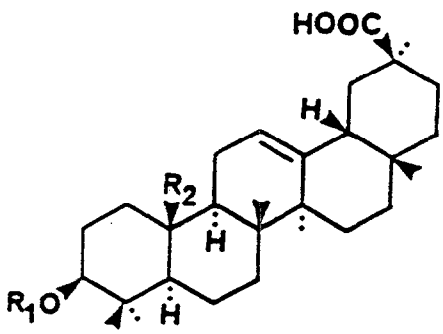
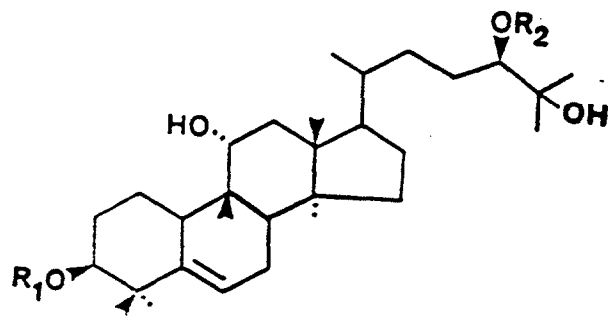


	R_1	R_2
2	β-glcA^2-β-glcA	CHO
3	β-glcA^2-β-glcA	CH_2OH



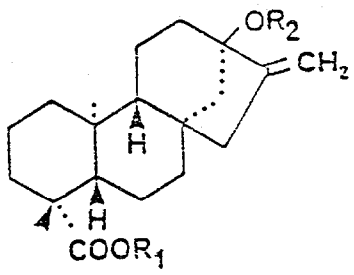
	R_1	R_2
4	β-glcA^2-β-glcA	CHO
5	β-glcA^2-β-glcA	CH_2OH



R_1	R_2
β-glc^6-β-glc	β-glc^6-β-glc |2 β-glc

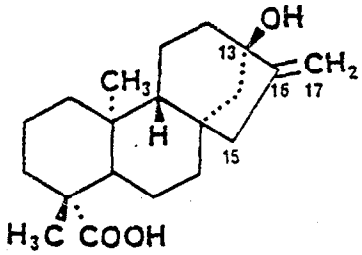
6

Figure I. Structures of hernandulcin (1), periandrins I-IV (2-5, respectively) and mogroside V (6), (GlcA = D-glucuronopyranosyl; glc = D - glucopyranosyl).



	R_1	R_2
7	β-glc	β-glc^2-β-glc
8	H	β-glc^2-β-glc
9	β-glc	β-glc^2-β-glc |3 β-glc
10	H	β-glc^2-β-glc |3 β-glc
11	β-glc	β-glc^2-α-rha |3 β-glc
12	β-glc^2-β-glc	β-glc^2-β-glc |3 β-glc
13	β-glc^2-β-glc	β-glc^2-β-glc
14	β-glc	β-glc^2-α-rha

Figure II. Structures of the sweet Stevia rebaudiana glycosides stevioside (7) steviolbioside (8), rebaudiosides A-E (9-13, respectively), and dulcoside A (14). (Glc = D-glucopyranosyl ; rha = L-rhamnopyranosyl)



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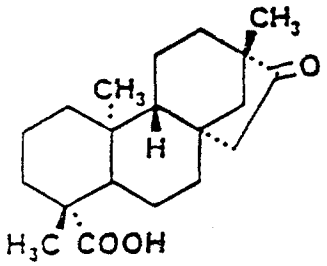

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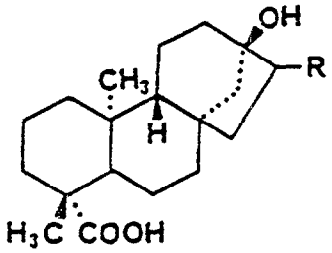
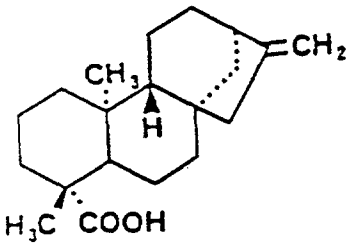


17 (R = β-CH_3)

18 (R = α-CH_3)



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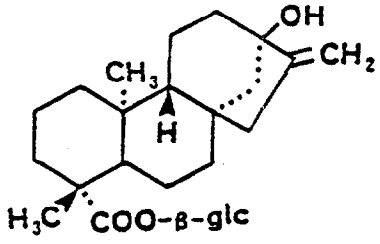

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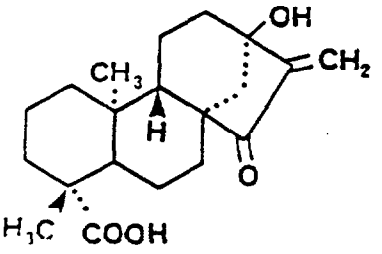

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Figure III. Structures of steviol (15), and its derivatives isosteviol (16), dihydrosteviol a (17) and B (18), ent-kaurenoic acid (19), 19-O-D-glucopyranosylsteviol (20), and 15-oxosteviol (21), tested for mutagenicity.

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Minor Cucurbitane-Glycosides from Fruits of *Siraitia grosvenori* (Cucurbitaceae)

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From fruits of *Siraitia grosvenori*, a Chinese medicinal plant, a new minor glycoside and four known minor cucurbitane glycosides, siamenoside I (sweet), 11-oxo-mogroside V (sweet), and mogrosides IIE and IIIE (both tasteless) were isolated together with mogrosides IV and V (both sweet) previously isolated from this fruit by Arihara *et al.* Structure of the new tasteless glycoside called mogroside III was elucidated as 3-*O*-β-D-glucopyranosyl-24-*O*-β-gentiobiosyl-mogrol. The relative sweetness of siamenoside I to sucrose was estimated ×563, making this the sweetest compound among the cucurbitane-glycosides so far isolated. The structure–taste relationship of cucurbitane-glycosides is also described.

Keywords *Siraitia grosvenori*; *Momordica grosvenori*; Cucurbitaceae; sweet principle; cucurbitane-glycoside; Chinese medicinal plant; mogroside; siamenoside

A fruit of *Siraitia grosvenori* SWINGLE (*Momordica grosvenori* SWINGLE., 羅漢果, Cucurbitaceae) growing in Kwangshi, China is used as an expectorant as well as a natural sweet food in that country. Two major sweet cucurbitane-glycosides called mogrosides IV (**1**) and V (**2**) have been isolated by Takemoto, Arihara *et al.*[1-3] Very recently, in our serial studies on Chinese cucurbitaceous medicinal plants, two new minor sweet glycosides called siamenoside I (**3**) and 11-oxo-mogroside V (**4**)[4] were isolated together with **1** and **2** from *Siraitia siamensis* CRAIB (翅子羅漢果) collected in South-Yunnan, China. The present paper reports the isolation and identification of minor cucurbitane glycosides from fruits of *Siraitia grosvenori*. The structure–taste relationship of glycosides of this type is also described.

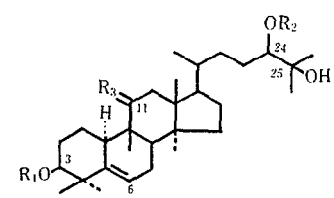
The dried fruits were extracted with methanol. A suspension of methanol-extract in water was defatted with hexane and then chromatographed on a highly porous synthetic polymer, Diaion HP-20. The fractions eluted with 50% and 80% methanol were respectively separated by repeated chromatography to give seven glycosides, A—G in yields of 0.025%, 0.008%, 0.029%, 0.044%, 0.034%, 0.18% and 0.45%, respectively.

Glycosides D, E, F and G were identified as **3**, **1**, **4** and **2**, respectively. Glycosides A and C were identified as mogrosides IIE (**5**) and IIIE (**6**), respectively, which were obtained from **2** by partial hydrolysis with maltase.[3] This is the first example of the occurrence of **5** and **6** in nature.

Comparison of the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of a new glycoside B (**7**) called mogroside III with that of mogrol (**8**), the common aglycone of **1**, **2** and **3**, showed the glycosylation shifts[5] for C-2, C-3 and C-24 as in the case of **2**, indicating that **7** is 3- and 24-diglycosyl-mogrol. On acid hydrolysis, **7** yielded D-glucose and the coupling constants of three anomeric proton signals demonstrated anomeric configurations of three glucopyranosyl units to be β. In the electron impact mass spectrum (EI-MS) of acetylated **7**, the fragment ions associated with terminal glycosyl (*m/z* 331) and glycobiosyl units (*m/z* 619) were observed. The sequencing analysis of permethylated **7** revealed the presence of terminal and 6-linked glucopyranosyl residues.[6] These results indicated the presence of β-D-glucosyl and β-gentiobiosyl residues in **7**.

The allocation of β-D-glucosyl and β-gentiobiosyl groups on the aglycone (**8**) was elucidated by means of the nuclear Overhauser effect (NOE) spectrum of acetylated **7**. The assignment of signals due to three sets of β-glucosyl protons of acetylated **7** was established by means of ^{1}H–^{1}H two dimensional correlation spectroscopy (2D COSY) as summarized in Table I. In the NOE spectrum, cross peaks were observed between an anomeric proton at δ 4.98 (1H, d, J=8.0 Hz) and 6-H$_2$ signals at δ 3.65 (1H, dd, J=7.3, 12.0 Hz) and 3.74 (1H, dd, J=2.2, 12.0 Hz), and were proved by ^{1}H–^{1}H 2D COSY to be located in the same glucosyl unit as that of an anomeric proton which appeared at δ 4.84 (1H, d, J=7.6 Hz). Accordingly, signals at δ 4.84 and 4.98 were assigned as anomeric protons of inner and terminal β-glucosyl units of a β-gentiobiosyl moiety, respectively. It follows that the remaining signal at δ 4.71 (1H, d, J=8.0 Hz) is assigned as an anomeric proton of an unsubstituted β-glucosyl unit. In the NOE spectrum, cross peaks were observed between the signal at δ 4.84 and the signal due to H-24 of the aglycone moiety at δ 3.48 (1H, dd, J=10.5, 5.4 Hz), and also between the signal at δ 4.71 and the signal due to H-3 of the aglycone moiety at δ 3.42 (1H, brs). Based on these results, **7** was formulated as 3-*O*-β-D-glucopyranosyl-24-*O*-β-gentiobiosyl-mogrol.

The relative sweetness to sucrose was determined in an 0.012% aqueous solution for **1** and **2**, 0.01% solution for **3** and 0.05% solution for **4** by a panel of five professional tasters in the manner described previously.[7] This panel also determined taste quality using an aqueous solution of



Glc: β-D-glucopyranosyl

	R_1	R_2	R_3
1	-Glc^{6}Glc	-Glc^{2}Glc	···OH / H
2	-Glc^{6}Glc	-Glc^{2}Glc (6-Glc)	···OH / H
3	-Glc	-Glc^{2}Glc (6-Glc)	···OH / H
4	-Glc^{6}Glc	-Glc^{2}Glc (6-Glc)	=O
5	-Glc	-Glc	···OH / H
6	-Glc	-Glc^{2}Glc	···OH / H
7	-Glc	-Glc^{6}Glc	···OH / H
8	-H	-H	···OH / H

TABLE I. ^{1}H-NMR Chemical Shifts (δ) for Sugar Moiety of Acetylated **7** in Acetone-d_6[a]

3-O-Sugar		24-O-Sugar			
		Inner		Terminal	
H-1	4.71 (d, J=8.0)	H-1	4.84 (d, J=7.6)	H-1′	4.98 (d, J=8.0)
H-2	4.82 (dd, J=8.0, 9.8)	H-2	4.89 (dd, J=7.6, 9.6)	H-2′	4.84 (dd, J=8.0, 9.6)
H-3	5.18 (dd, J=9.3, 9.8)	H-3	5.19 (dd, J=9.3, 9.6)	H-3′	5.21 (dd, J=9.6, 9.6)
H-4	4.91 (dd, J=9.3, 10.2)	H-4	4.82 (dd, J=9.3, 10.2)	H-4′	4.94 (dd, J=9.6, 10.2)
H-5	3.85 (ddd, J=2.5, 5.5, 10.2)	H-5	3.87 (ddd, J=2.2, 7.3, 10.2)	H-5′	3.86 (ddd, J=2.5, 5.2, 10.2)
H-6	4.04 (dd, J=2.5, 12.0)	H-6	3.65 (dd, J=7.3, 12.0)	H-6′	4.06 (dd, J=2.5, 12.2)
	4.18 (dd, J=5.5, 12.0)		3.74 (dd, J=2.2, 12.0)		4.19 (dd, J=5.2, 12.2)

a) Measured at 400 MHz with TMS as the internal standard; coupling constants are given in Hz (d, doublet).

TABLE II. Taste and Relative Sweetness

Compound	Conc.[a] (%)	Taste	Relative sweetness[b]
1	0.012	Sweet	392
2	0.012	Sweet	425
3	0.010	Sweet	563
4	0.05	Sweet	84
5		Tasteless	
6		Tasteless	
7		Tasteless	

a) Concentration of sample in aqueous solution (w/v%). b) Sucrose = 1.

samples at a concentration which exhibited similar sweetness to that of a 5% aqueous solution of sucrose. The results are summarized in Table II.

We previously isolated a number of cucurbitane glycosides from the rhizomes of *Hemsleya carnosiflora* C. Y. Wu *et* Z. L. Chen[8] and *H. panacis-scandens* C. Y. Wu *et* Z. L. Chen[6] collected in Yunnan, China. In those studies on the structure–taste relationship of these compounds and their derivatives, it was suggested that the oxygen function at the 11-position of the aglycone moiety is responsible for the occurrence of taste; glycosides of 11α-hydroxy-compounds taste sweet, while glycosides of 11β-hydroxy-compounds are tasteless and 11-keto-glycosides taste bitter.[6,8] In the present study, glycosides of 11α-hydroxy-aglycone, **1**, **2** and **3** were found to taste very sweet, while a remarkable decrease in sweetness was observed for the glycoside (**4**) of 11-keto-aglycone. The quality of taste of **4** was also significantly poorer than **2** and **3**.

It has been observed that the number of glucose units is also responsible for the occurrence of taste.[6,8] Mogrol-glycosides, **5**, **6** and **7** which have less than three glycosyl units, are almost tasteless. The relationship between the allocation of glucosyl units and sweetness is also noteworthy. Siamenoside I (**3**) which has four glucosyl units, is the sweetest compound among glycosides of this type so far isolated and shows a similar good the taste quality to **2** which has five glucosyl units, while the sweetness of mogroside IV (**1**) with the same number of glucosyl units as **3** is even less than **3**.

Experimental

Optical rotations were measured with a Union PM-101 automatic digital polarimeter and a Jasco DIP-360 digital polarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL GX-400 spectrometer in C_5D_5N solution (acetates of **7**: in acetone-d_6) using tetramethylsilane (TMS) as an internal standard. EI-MS and fast atom bombardment mass spectrum (FAB-MS) were recorded on a JEOL SX-102 spectrometer. Gas liquid chromatography mass spectrometry (GC-MS) were recorded on a Hitachi M-80B mass spectrometer. Acetylation procedure for the EI-MS and ^{1}H-NMR: A solution of a few mg of glycoside in Ac_2O (1 ml) and C_5H_5N (2 ml) was allowed to stand at room temperature overnight. After work-up in the usual way, the resulting acetate was subjected to spectrometry. High performance liquid chromatography (HPLC) was carried out with a Tosoh CCPM pump equipped with a Tosoh UV 8010 UV/VIS as a detector on a YMS packed column D-ODS-R (20 mm × 250 cm), flow rate: 5 ml/min. For column chromatography, silica gel: Kieselgel 60 (Merck, 70—230 mesh), silanized silica gel: Cosmosil 75C_{18}-OPN (Nacalai Tesque) and highly porous synthetic resin: Diaion HP-20 (Mitsubishi Chem. Ind. Co., Ltd.) were used. All solvent systems for chromatography were homogeneous. Identification of known compounds was made by comparison of the ^{1}H- and ^{13}C-NMR spectra and optical rotation with those of a respective authentic sample. In the case of **5** and **6**, identification was established by comparison of the EI-MS, ^{1}H- and ^{13}C-NMR spectra of their acetates and $[\alpha]_D$ with the reference data. Acid hydrolysis of glycosides and identification of the resulting monosaccharides including absolute configuration were carried out as reported previously.[6] Permethylation and the sequencing analysis of sugar moiety were also referred to in the previous paper.[6]

Extraction and Separation The dried fruits of *Siraitia grosvenori* (240 g) purchased in Macao were extracted with MeOH. A suspension of the MeOH-extract (34 g) in H_2O was defatted with C_6H_{12}. The H_2O layer was successively chromatographed on Diaion HP-20 with H_2O, 50% MeOH, 80% MeOH, MeOH and $(CH_3)_2CO$.

The 50% MeOH eluate was separated by column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (6:4:1) and then successively on silanized silica gel eluted with 56% and 70% MeOH to give **4** and **2**.

The 80% MeOH eluate was chromatographed on silica gel with $CHCl_3$–MeOH–H_2O (6:4:1) to give fractions I—IX. Fraction I was subjected to HPLC; mobile phase: 62% MeOH to give **5**. Fraction IV was subjected to chromatography on silica gel with $CHCl_3$–MeOH–H_2O (10:5:1) followed by HPLC; mobile phase: 65% MeOH to give **7**. Fraction V was separated in the same way as fraction IV, yielding **6**. Fraction VII was chromatographed on silanized silica gel with 60% MeOH to afford **3** and **1**. Chromatography of fraction VIII on silanized silica gel with 60% MeOH afforded **1** and **4**. Fraction IX was chromatographed on silica gel with $CHCl_3$–MeOH–H_2O (6:4:1) and then on silanized silica gel with 56% MeOH to give **2**.

Mogroside IV (**1**): A white powder, $[\alpha]_D^{22}$ −5.8° (c=1.04, MeOH). IR (Nujol) cm^{-1}: 3400 (OH), 1640, 890 (C=C). ^{1}H-NMR δ: anomeric H 5.34 (1H, d, J=7.7 Hz), 5.17 (1H, d, J=7.9 Hz), 5.09 (1H, d, J=7.7 Hz), 4.82 (1H, d, J=7.9 Hz). ^{13}C-NMR δ: anomeric C 107.0, 106.4, 105.5, 101.8.

Mogroside V (**2**): A white powder, $[\alpha]_D^{22}$ −11.7° (c=1.02, MeOH). IR (Nujol) cm^{-1}: 3400 (OH), 1640, 890 (C=C). ^{1}H-NMR δ: anomeric H 5.41 (1H, d, J=7.7 Hz), 4.88 (1H, d, J=8.6 Hz), 4.86 (1H, d, J=6.1 Hz), 4.84 (1H, d, J=7.4 Hz), 4.79 (1H, d, J=7.7 Hz). ^{13}C-NMR δ: anomeric C 106.8, 105.5, 105.2, 104.7, 103.5.

Siamenoside I (**3**): A white powder, $[\alpha]_D^{22}$ +4.9° (c=1.03, MeOH). IR (Nujol) cm^{-1}: 3400 (OH), 1640, 890 (C=C). ^{1}H-NMR δ: anomeric H 5.38 (1H, d, J=7.7 Hz), 4.99 (1H, d, J=7.7 Hz), 4.86 (1H, d, J=7.7 Hz), 4.84 (1H, d, J=7.5 Hz). ^{13}C-NMR δ: anomeric C 107.3, 105.7, 104.9, 103.6.

11-Oxo-mogroside V (**4**): A white powder, $[\alpha]_D^{25}$ +20.5° (c=0.51, MeOH). IR (Nujol) cm^{-1}: 3400 (OH), 1640, 890 (C=C). ^{1}H-NMR δ: anomeric H 5.46 (1H, d, J=7.7 Hz), 5.12 (1H, d, J=7.7 Hz), 4.88 (1H, d, J=7.9 Hz), 4.84 (1H, d, J=7.7 Hz), 4.77 (1H, d, J=7.5 Hz). ^{13}C-NMR δ: anomeric C 106.9, 105.6, 105.5, 104.9, 103.6.

Mogroside IIE (**5**): A white powder, $[\alpha]_D^{23}$ +35.2° (c=0.88, MeOH). IR (Nujol) cm^{-1}: 3400 (OH), 1640, 890 (C=C). ^{1}H-NMR δ: anomeric H 4.98 (1H, d, J=8.0 Hz), 4.87 (1H, d, J=7.8 Hz). ^{13}C-NMR δ: anomeric

C 107.0, 105.8.

Mogroside IIIE (**6**): A white powder, $[\alpha]_D^{23}$ +4.5° (c=0.88, MeOH). IR (Nujol) cm^{-1}: 3400 (OH), 1640, 890 (C=C). ^{1}H-NMR δ: anomeric H 5.28 (1H, d, J=7.7 Hz), 5.03 (1H, d, J=7.7 Hz), 4.84 (1H, d, J=7.7 Hz). ^{13}C-NMR δ: anomeric C 107.2, 106.2, 101.7.

Mogroside III (**7**): A white powder, $[\alpha]_D^{20}$ +2.5° (c=0.36, MeOH). High resolution FAB-MS m/z: Calcd for $C_{48}H_{82}O_{19}$+Na 985.5349. Found 985.5344 $(M+Na)^+$. IR (Nujol) cm^{-1}: 3400 (OH), 1640, 890 (C=C). ^{1}H-NMR δ: anomeric H 4.86 (1H, d, J=7.9 Hz), 4.84 (1H, d, J=7.7 Hz), 4.80 (1H, d, J=7.5 Hz). ^{13}C-NMR δ: aglycone moiety 26.2 (C1), 29.5 (C2), 87.9 (C3), 42.4 (C4), 144.2 (C5), 118.5 (C6), 24.6 (C7), 43.5 (C8), 40.1 (C9), 36.9 ((C10), 77.8 (C11), 41.1 (C12), 47.4 (C13), 49.7 (C14), 34.6 (C15), 28.2 (C16), 51.1 (C17), 17.1 (C18),[a] 26.7 (C19),[a] 36.2 (C20), 18.8 (C21), 33.1 (C22), 27.5 (C23), 92.8 (C24), 72.7 (C25), 24.2 (C26),[a] 26.3 (C27),[a] 19.3 (C28), 27.7 (C29),[a] 26.3 (C30),[a] glucosyl moiety 107.4 (C1), 75.5 (C2),[b] 78.5 (C3),[c] 71.5 (C4),[d] 78.1 (C5)[c] 63.1 (C6),[e] gentiobiosyl moiety: inner Glc 104.8 (C1), 75.1 (C2), [b] 78.1 (C3),[c] 72.1 (C4),[d] 76.4 (C5), 70.4 (C6), terminal Glc 106.3 (C1), 75.5 (C2),[b] 78.7 (C3),[c] 71.8 (C4),[d] 78.6 (C5),[c] 62.5 (C6).[e] (a—l: are interchangeable).

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Note

Sweet Cucurbitane Glycosides from Fruits of *Siraitia siamensis* (chi-zi luo-han-guo), a Chinese Folk Medicine

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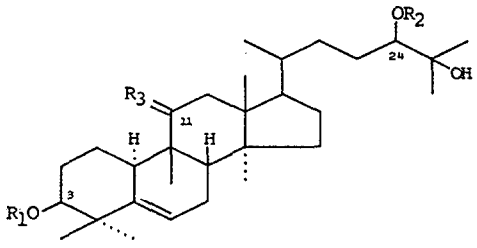
Cucurbitane-type triterpenes are known as bitter principles of cucurbitaceous plants, while some of the glycosides of triterpenes of this type are sweet tasting; bryodulcoside[1] from roots of *Bryonia dioica* JACQ., mogrosides IV (**1**) and V (**2**)[2~4] from fruits of *Siraitia grosvenori* SWINGLE (=*Momordica grosvenori* SWINGLE; Chinese name of the fruit, luo-han-guo) and glycosides from rhizomes of *Hemsleya carnosiflora* C. Y. WU *et* Z. L. CHEN sp. nov.[5] and *H. panacis-scandens* C. Y. WU *et* Z. L. CHEN.[6] In our series of studies on Chinese cucurbitaceous plants, the present paper describes the characterization of sweet glycosides from fruits of *Siraitia siamensis* CRAIB. (Chinese name, chi-zi lou-han-guo) which grows in Xi-shuang-bana, South-Yunnan, China and is closely related to *S. grosvenori*.

Dried and powdered fruits were extracted with petroleum ether to remove the lipophilic materials and then extracted with methanol. The sweet methanolic extract was separated by chromatography on a highly porous polymer, then on silica gel and finally by high-performance liquid chromatography (HPLC) on a reverse-phase column to give six glycosides, A-F, in yields of 0.036, 0.57, 0.047, 0.12, 0.013 and 0.055%, respectively.

Glycosides B and D were identified as **2** and **1**, respectively, both of which have already been isolated from Luo-han-guo by Takemoto *et al.*[2~4]

A new sweet glycoside A (**4**) afforded D-glucose on acid hydrolysis. The ^{1}H- and ^{13}C-NMR spectra of **4** exhibited signals due to anomeric protons and carbons of five glucoside units (see the experimental section).

The ^{1}H- and ^{13}C-NMR signals of mogrol (**3**), which is the common aglycone of **1** and **2**, were characterized by means of ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY and 2D-INADEQUATE procedures as shown in Tables I and II. In a comparison of the ^{13}C-NMR spectra of **1** and **3**, the signal due to the 24-carbinyl carbon was displaced downfield by 9.0 ppm on glycosylation (glycosylation



	R_1	R_2	R_3
1	-Glc^{6}—Glc	-Glc^{2}—Glc	H, OH
2	-Glc^{6}—Glc	-Glc^{2}(6-Glc)—Glc	H, OH
3	-H	-H	H, OH
4	-Glc^{6}—Glc	-Glc^{2}(6-Glc)—Glc	=O
5	-H	-H	=O
6	-Glc	-Glc^{2}(6-Glc)—Glc	H, OH

Glc: β-D-glucopyranosyl

Chart 1.

Table I. ^{1}H-NMR SPECTRAL DATA OF MOGROL (3)

(400 MHz, d_6-acetone with TMS)

H		
3	3.40 t	J=2.7 Hz
6	5.47 d	J=6.1 Hz
7α	1.78 dd	J=10.4, 6.1 Hz
8	1.67 d	J=7.3 Hz
10	2.55 br. d	J=12.1 Hz
11	3.87 dd	J=11.4, 5.5 Hz
17	1.59 br. t	J=8.2 Hz
18	0.91 s	
21	0.95 d	J=6.2 Hz
22	1.30 dd	J=9.4, 1.7 Hz
23	1.35 br. d	J=10.3 Hz
24	3.25 dd	J=10.3, 2.0 Hz

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Table II. ^{13}C-NMR SPECTRAL DATA OF THE AGLYCONE MOIETIES OF **1**~**6**

(100 MHz, C_5D_5N with TMS)

C-No.	3	1	2	6	5	4
1	25.8	26.8	26.8	26.8	21.2	22.2
2	30.8	29.4	29.4	29.4[b]	29.7	29.2
3	76.2	87.4	87.4	87.9	75.5	86.6
4	42.2	42.3	42.2	42.3	41.8	41.9
5	144.3	144.3	144.3	144.2	141.3	141.2
6	119.1	118.4	118.4	118.4	118.9	118.4
7	24.5	24.5	24.6	24.5	24.2	24.0
8	43.6	43.5	43.5	43.5	44.0	44.0
9	40.2	40.1	40.1	40.1	49.1	49.0
10	36.9	36.7	36.7	36.7	35.9	35.9[b]
11	77.8	77.9	77.9	77.9	213.8	213.9
12	41.2	41.0	41.0	41.1	48.7	48.8
13	47.4	47.4	47.4	47.4	49.1	49.0
14	49.8	49.7	49.7	49.6	49.6	49.7
15	34.5	34.5	34.5	34.5	34.5	34.5
16	28.4	28.5	28.5	28.5	28.1	28.3
17	51.0	50.9	51.0	51.0	49.8	49.9
18	17.0	17.0	17.1	17.1	16.9[a]	17.0[a]
19	26.7[a]	27.0	27.0	27.0	20.1	20.3
20	36.3	36.7	36.3	36.3	35.9	36.2[b]
21	18.9	19.1	19.1	19.0	18.2[a]	18.3[a]
22	34.2	33.8	33.2	33.2	33.9	33.0
23	29.0	28.5	29.4	29.5[b]	28.6	28.5
24	79.0	88.0	91.9	92.1	78.9	92.0
25	72.7	72.4	72.7	72.8	72.7	72.7
26	25.8[a]	25.8[a]	24.6[a]	24.5[a]	25.9	25.8
27	26.2[a]	26.2[a]	26.2[a]	26.3[a]	26.1	24.6
28	19.3[a]	19.3[a]	19.4[a]	19.4[a]	18.5[a]	18.7[a]
29	27.3	27.6	27.6	27.6	27.9	28.2
30	26.2[a]	26.2[a]	26.2[a]	26.3[a]	27.0[a]	27.0[a]

[a,b] These signals may be interchanged in each column.

shift).[7~9] On the other hand, an unexpectedly large glycosylation shift (+12.9 ppm) of the 24-C signal was observed for **2** which had a bulky sugar moiety (branched chain) at the 24-hydroxyl group. This anomalous shift is useful for allocating the sugar moiety of the related glycosides.

A comparison of aglycone carbon signals for **4** with those of 11-oxomogrol[3] (=bryoducosigenin, **5**[11]) and **2** (Table II) indicated that **4** must be a 3,24-*O*-bisglycoside of **5**. The carbon signals due to the sugar moiety of **4** were almost superimposable on those of **2**. The anomalous glycosylation shift of 24-C (*vide supra*) was observed from **5** to **4**, leading to the structure of 11-oxomogroside V as shown in Chart 1.

A new sweet glycoside C named siamenoside I (**6**) afforded D-glucose after acid hydrolysis. The ^{1}H- and ^{13}C-NMR spectra showed signals due to anomeric protons and carbons of four β-glucoside units (see the experimental section). The EI-MS of an acetate (**7**) of **6** exhibited fragment ions due to Glc-Ac_4 (m/z 331) and Glc_3-Ac_{10} (m/z 907) but no fragment ion due to Glc_2-Ac_8 (m/z 619) suggesting the presence of a branched chain glucotrioside moiety like that in **2**. All of the carbon signals due to the aglycone moiety of **6** appeared at very similar chemical shifts to those of **2**, including the anomalous glycosylation shift of 24-C (Table II). These results indicated that **6** must be 3-*O*-β-glucosyl-24-*O*-glucotriosyl (branched)-mogrol. The structure of the 24-*O*-glucotriosyl moiety of **6** was elucidated by means of the ^{1}H-^{1}H COSY and ^{1}H-^{1}H NOESY spectra (in acetone-d_6) of **7**. In the ^{1}H-NMR spectrum of **7**, most of the carbinyl proton signals of the glycosyl moiety appeared downfield by acetylation shift, while signals which were not displaced downfield [δ3.71 (1H, dd, J=7.7, 8.3 Hz) and δ3.68 (1H, dd, J=4.0, 12.1 Hz) and 3.79 (1H, dd, J=2.3, 12.1 Hz)] were respectively characterized as 2-H and 6-H_2 in the same glucoside unit based on the ^{1}H-^{1}H COSY procedure. The presence of NOE between this 2-H signal and an anomeric proton signal at δ4.51 (1H, d, J=7.9 Hz), as well as between one of the 6-H_2 signals (δ3.68, *vide supra*) and an anomeric proton signal at δ4.79 (1H, d, J=8.1 Hz), was substantiated by the ^{1}H-^{1}H NOESY procedure. These results indicated the presence of a 2,6-di-*O*-β-glucosyl-β-glucoside moiety. A signal at δ4.45 (1H, d, J=7.7 Hz) was assigned as an anomeric proton of the foregoing 2,6-linked glucosyl unit by the ^{1}H-^{1}H COSY procedure. The presence of NOE between this anomeric proton signal and the 24-carbinyl proton signal of the aglycone moiety at δ3.40 (1H, br. d, J=8.3 Hz) was observed by the ^{1}H-^{1}H NOESY procedure. This revealed the allocation of the 2,6-linked glucoside unit at the 24-hydroxyl group. The presence of NOE between a signal due to the 3-carbinyl proton of the aglycone moiety at δ3.34 (1H, br. s) and a remaining anomeric proton signal at δ4.60 (1H, d, J=8.0 Hz) was also observed by the ^{1}H-^{1}H NOESY procedure. Thus, **6** could be formulated as mogrol-3-*O*-β-D-glucopyranosido-24-*O*-[β-D-glucopyranosyl(1→2)]-[β-D-glucopyranosyl-(1→6)]-β-D-glucopyranoside.

A structural elucidation of compounds E and F has not yet been done due to the shortage of materials.

Experimental

NMR spectra were recorded with a JEOL GX 400 spectrometer at 400 MHz for protons and at 100 MHz for carbon-13 in C_5D_5N unless otherwise stated.

Plant material. The plant was collected at Xi-shuang-ba-na, South Yunnan, China and identified by Emeritus Professor C. Y. Wu of the Kunming Institute of Botany, Chinese Academy of Science. A voucher specimen has been deposited in the herbarium of this institute.

Conditions for preparative HPLC. Reverse-phase column, TSK-GEL ODS-120T (21 mm i.d. × 30 cm); detection, R.I. and UV (210 nm); flow rate, 6 ml/min.

Acid hydrolysis and subsequent identification of the resulting monosaccharide. A solution of a few milligram of each glycoside in 1 N H_2SO_4/50% EtOH (1 ml) was heated at 100 C in a sealed tube for 4 hr. The reaction mixture was treated with $BaCO_3$ and then with Amberlite MB-3, and concentrated to dryness. The residue was subjected to the identification of monosaccharides including the absolute configuration that was reported by Ohshima *et al.*[10] Each compound (**1**, **2**, **4**, **6**, and glycosides E and F) afforded D-glucose.

Extraction and separation. Dried and powdered fruits (207 g) were defatted by extracting with petroleum ether and then extracted with hot MeOH. This extract (65 g) was chromatographed on highly porous polymer resin (DA-201, made in China) by eluting with H_2O and then with 70% MeOH. The 70% MeOH eluate was dissolved in MeOH to remove the MeOH-insoluble substances to give an MeOH-soluble sweet fraction (10.5 g), which was chromatographed on silica gel by eluting with $CHCl_3$–MeOH (9:1 then 5:5) to separate into seven fractions (I ~ VII). A part (1.5 g) of fraction V (2.7 g) was further chromatographed on silica gel by eluting with $CHCl_3$–MeOH–H_2O (6:4:1, homogeneous) to give fractions V-1, -2 and -3. Fraction V-2 (544 mg) was subjected to HPLC (mobile phase, 57% MeOH) to give **2** (140 mg) and **6** (54 mg). Fraction V-3 (917 mg) was separated by chromatography on silica gel [$CHCl_3$–MeOH–H_2O solvent (6:4:1, homogeneous)] and followed by HPLC (54% MeOH mobile phase) to give **1** (372 mg) and glycoside E (15 mg).

A part (503 mg) of fraction VI (910 mg) was separated by chromatography on silica gel [$CHCl_3$–MeOH–H_2O solvent (6:4:1, homogeneous)] and followed by HPLC (mobile phase, 54% MeOH) to give **4** (42 mg) and **1** (270 mg). A part (503 mg) of fraction VII (1.24 g) was subjected to chromatography on silica gel by eluting with $CHCl_3$–MeOH–H_2O (6:4:1, homogeneous) and followed by HPLC (mobile phase, 56% MeOH) to give glycoside F (60 mg).

1 (mogroside IV): a white powder, $[\alpha]_D^{18}$ −4.4° (c=0.91, MeOH), NMR δ_H anomeric H: 4.81 (1H, d, J=7.6 Hz), 5.07 (1H, d, J=7.3 Hz), 5.16 (1H, d, J=7.6 Hz), 5.33 (1H, d, J=7.3 Hz). **2** (mogroside V): a white powder, $[\alpha]_D^{23}$ −6.3° (c=0.50, H_2O), NMR δ_H anomeric H: 4.80 (1H, d, J=7.8 Hz), 4.86 (1H, d, J=7.5 Hz), 4.89 (1H, d, J=7.8 Hz), 5.15 (1H, d, J=7.8 Hz), 5.47 (1H, d, J=7.5 Hz). Identification of **1** and **2** was conducted by comparing the optical rotation, and ^{1}H- and ^{13}C-NMR spectra with those of respective authentic samples. **4**: A white powder, $[\alpha]_D^{18}$ +24.3° (c=1.07, MeOH). *Anal.* Found: C, 53.41; H, 7.79%. Calcd. for $C_{60}H_{100}O_{29}$ 7/2 H_2O: C, 53.44; H, 8.00%. NMR δ_H anomeric H: 4.81 (1H, d, J=8.6 Hz), 4.86 (1H, d, J=7.6 Hz), 4.90 (1H, d, J=7.3 Hz), 5.13 (1H, d, J=7.9 Hz), 5.49 (1H, d, J=7.3 Hz); δ_C anomeric C: 103.6 (1C), 104.8 (1C), 105.4 (2C), 106.9 (1C). **6**: A white powder, $[\alpha]_D^{18}$ +3.5° (c=0.52, MeOH). *Anal.* Found: C, 54.58; H, 8.10%. Calcd. for $C_{54}H_{92}O_{24}$ 7/2 H_2O: C, 54.58; H, 8.40%. NMR δ_H anomeric H: 4.86 (1H, d, J=7.6 Hz), 4.87 (1H, d, J=8.0 Hz), 4.91 (1H, d, J=7.3 Hz), 5.47 (1H, d, J=7.6 Hz); δ_C anomeric C: 103.7 (1C), 104.8 (1C), 105.4 (1C), 107.3 (1C).

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Antioxidant Property of Fructus Momordicae Extract

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ABSTRACT

The antioxidant effect of Fructus Momordicae extract, FME (mogrosides 75~80%), was studied. FME reduced the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) and scavenged superoxide radicals (O_2^-) generated by a hypoxanthine and xanthine oxidase system. It also scavenged hydroxyl radicals (•OH) generated by Fenton reaction. In addition, FME inhibited Fe(II) induced lipid peroxidation in rat cortex homogenates in a dose-dependent manner, as indicated by decreased thiobarbituric acid-reactive substances (TBARS) formation. Oral administration of FME inhibited TBARS and malonaldehyde (MDA) formation in the ipsilateral cortex 30 min after iron-salt injection into the left cortex of rat. FME showed inhibitory effect on 4-hydroxy-2(E)-nonenal (4-HNE) formation induced by Fe(III) injection into the rat cortex. These data suggest that Fructus Momordicae extract has an antioxidant activity against free radicals and lipid peroxidation.

KEY WORDS: Fructus Momordicae extract; Antioxidant; Free radicals; Lipid peroxidation; Post-traumatic epilepsy; Fe(II); Fe(III); TBARS; MDA and 4-HNE.

INTRODUCTION

A variety of clinical pathological events have been found in recent years to be connected with oxygen free radical injuries (1-6). Free radicals can react with vital cellular components such as nucleic acids, proteins and membranes, leading to the disturbances in structure and function of

Abbreviation

FME, Fructus Momordicae extract; DETAPAC, diethylenetriaminepentaacetic acid; DMPO, 5,5-Dimethyl-1-pyrroline-N-oxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ESR, electron spin resonance; 4-HNE,4-hydroxynonenal; MDA, malonaldehyde; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; XOD, xanthine oxidase; PBS, phosphate buffer solution.

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the cells. Lipid peroxidation is a deleterious effect induced by oxygen free radicals. Brain tissue is more vulnerable to the toxic effects of free radicals in comparison with other organs of the body because of its high rate of oxidative metabolism and high concentration of readily oxidizable substrates, such as membrane lipid polyunsaturated fatty acids(7). It has been found that free radicals damage neuronal membranes, and thus induce and propagate lipid peroxidation in various neurological disorders such as epilepsy (8), ischemia (9,10), and trauma (11). Finding effective antioxidants may prove beneficial for treating these diseases and clarifying pathogenesis of these diseases.

Fructus Momordicae, the fruit of *Momordica Grosvenori* Swingle (a perennial classified to the cucurbitaceae family), has long been used for the treatment of acute and chronic bronchitis, gastritis, sore throats, minor stomach and intestinal troubles, and whooping cough in traditional Chinese medicine(12-14). Many reports showed that, pathologies of inflammation are related to free radical processes, and most medicines beneficial for inflammation show antioxidant properties(2,15-19). However, there is little information about the physiological action of Fructus Momordicae in this process. Chemical studies show that it contains sweet components which are called mogrosides(12,20,21), in addition to proteins, lipids and minerals etc. Most mogrosides in Fructus Momordicae are 300 times sweeter than sugar (21). Recently, the concentrated mogroside extracts of Fructus Momordicae have been obtained. The percent of sweet components in the extracts was 75% (FME-A) or 80% (FME-B).

In the present study, the free radical scavenging activity and effect on thiobarbituric acid reactive substances (TBARS) formation in rat cortex homogenates of FME-A and FME-B were examined in vitro. Furthermore the ability of FMEs to inhibit lipid peroxidation in vivo was tested. The effects of FMEs on TBARS, malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE) production in the $FeCl_3$-induced cortical epileptic model of rat were investigated.

MATERIAL AND METHODS

Chemicals 1,1-diphenyl-2-picrylhydrazyl (DPPH) was sourced from Sigma Chem. Co. (St. Louis, MO, U.S.A.). 5,5-Dimethyl-1-pyrroline-1-oxide(DMPO) was from Labotec Co., Ltd. (Tokyo, Japan). Diethylenetriaminepentaacetic acid (DETAPAC) was from Aldrich Chemical Company, Inc. (WI, U.S.A.). Xanthine oxidase (XOD, cow milk) was from Boeringer Mannheim GmbH (Germany). FMEs were kindly supplied by Clean Cycle Company, LTD. (Tokyo, Japan). All other chemicals used were of the highest grade available in Japan.

Animals Male Wistar rats weighing 200-220 g (Funabashi Farm Japan Inc., Sendai, Japan) were used for the experiments.

Lipid peroxidation assay in rat cortex homogenates Rat was decapitated and the cortex was rapidly removed and homogenized in 10 volumes of ice-cold phosphate buffer solution (PBS, pH 7.4) using a glass homogenizer, and centrifuged at 1,000 x g for 10 min. Protein concentration was assayed by the method of Lowry *et al.* (22). 0.1ml of two times diluted cortex homogenates, 0.04ml of 2mM $FeCl_2$, 0.02 ml of 0.2mM ascorbic acid and tested compounds were mixed well, and incubated at 37°C for 15 min. TBARS were measured according to the method of Ohkawa *et al.* by spectrofluorometry (EX: 515 nm, EM: 553 nm) (23).

Free radical analysis The analysis of each free radical was carried out by using ESR spectrometer (JOEL JES-FEIXG, Tokyo, Japan). Manganese oxide was used as an internal standard (24). (1) DPPH radical: 350µl of 30 µM DPPH ethanol solution and 50 µl of FME-A or FME-B suspended phosphate buffer solution were placed in a test tube and mixed for 10 sec. The mixture was transferred to a special flat cell (JOEL Co. Ltd., Tokyo, Japan) for DPPH analysis by ESR; the measurement was performed 60 sec after addition of each sample. The signal intensity was evaluated from the peak height of the 3rd signal of DPPH radicals. (2) O_2^-: 50 µl of FME-A or FME-B suspended phosphate buffer solution, 35 µl of 5.5 mM DETAPAC, 50µl of 2 mM hypoxanthine, 15 µl of 8.97 M DMPO and 50 µl of 0.4 U/ml xanthine oxidase were put into a test tube and mixed for 10 sec. The mixture was transferred to a special flat cell for the analysis of the amount of DMPO spin adducts of superoxide radicals (DMPO-OOH). The assay was performed 60 sec after addition of xanthine oxidase. The signal intensity was evaluated from the peak height of the first signal. (3) •OH: Seventy five microliters of 1 mM $FeSO_4$-DETAPAC solution, 50 µl of FMEs suspended solution, 20 µl of 0.92 M DMPO and 50 µl of 1 mM hydrogen peroxide were put into a test tube and mixed for 10 sec. The mixture was transferred to a special flat cell for the analysis of the amount of DMPO spin adducts of hydroxyl radicals (DMPO-OH). The assay was performed 60 sec after addition of hydrogen peroxide. The signal intensity was evaluated from the peak height of the first signal. The conditions of ESR spectrometer were as follows: Magnetic field, 335 ± 10 mT; field modulation width, 0.079 mT; receiver gain, 3.2 × 100; time constant, 0.1 sec; sweep time, 2 min; microwave power, 8mW.

Iron-induced epileptic model in rat

5µl of freshly prepared 100 mmol/L $FeCl_3$ solution was injected into the left cortex of rat over 5 min according to the method of Willmore et al.(25). Briefly, animals were anesthetized and placed in a stereotaxic apparatus. A burr hole was made in the left calvarium 1mm posterior and 1mm lateral to the bregma. A 25-gauge needle attached to a microsyringe firmly held in a stereotaxic micromanipulator was inserted into the left cortex to 2.5 mm below the exposed dura. Control animals were injected with 5 µl of saline adjusted to the pH of the $FeCl_3$ solution. One ml of FME-A or FME-B (50mg/ml or 100mg/ml) was administered orally to rats 30 min before injection of the $FeCl_3$ solution. Each rat was killed 30 min after completion of the injection of iron solution or saline. The brain was removed, and the cortex was dissected on a metal plate in contact with ice. Samples were stored at -80°C until the performance of TBARS, MDA and 4-HNE assay.

MDA and 4-HNE assay:

The assay was performed using the LPO-586 Test Kits from BIOXYTECH, OXIS International S.A. (94385 Bonneuil/Marne, FRANCE).

Statistical analysis

Calculations of difference of statistical significance were made using two-tail student's t-test.

RESULTS

In the DPPH test, the free radical scavenging activity of the tested drugs was expressed by IC_{50}. The scavenging activities of FMEs increased in a concentration-dependent manner and IC_{50} of FME-A and -B was 4.5μg/ml and 2.9μg/ml, respectively (Fig.1). In addition, kinetic study indicated that FMEs quenched DPPH radicals in a time-dependent manner. They could markedly decrease radical concentration as time progressed (data not shown). The ability of FMEs to scavenge superoxide radicals and hydroxyl radicals was measured by ESR spin trapping technique. FME-A and FME-B scavenged superoxide radicals (O_2^-) with IC_{50} of 0.44 mg/ml and 0.60 mg/ml, respectively (Fig. 2). Fe(II) and hydrogen peroxide system was used to generate hydroxyl radicals. FME-A and FME-B inhibited hydroxyl radicals (•OH) generated by Fenton reaction with IC_{50} of 0.83 mg/ml and 0.62 mg/ml, respectively (Fig. 3).

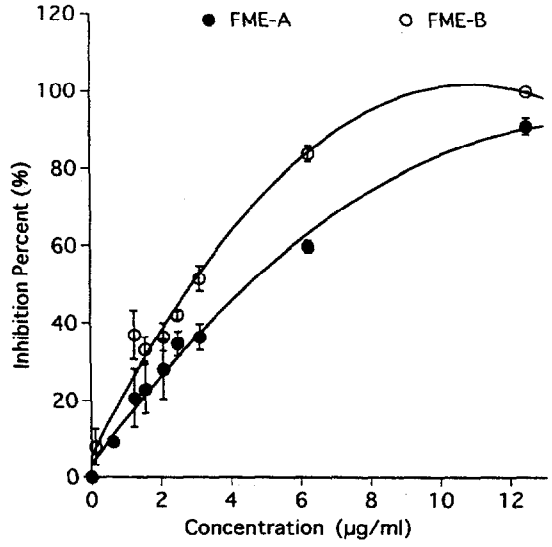

Fig. 1. Scavenging effect of FME-A and FME-B on 1,1-diphenyl-2-picrylhydrazyl radicals. Each bar represents the mean ± SD of 3 or 4 determinations.

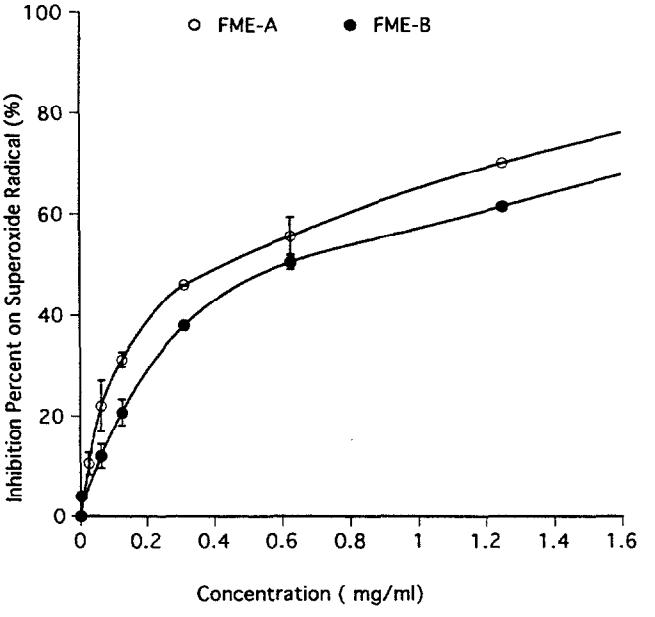

Fig. 2. Effect of FME-A and FME-B on superoxide (O_2^-) generated by hypoxanthine and xanthine oxidase system. Each bar represents the mean ± SD of 3 or 4 determinations.

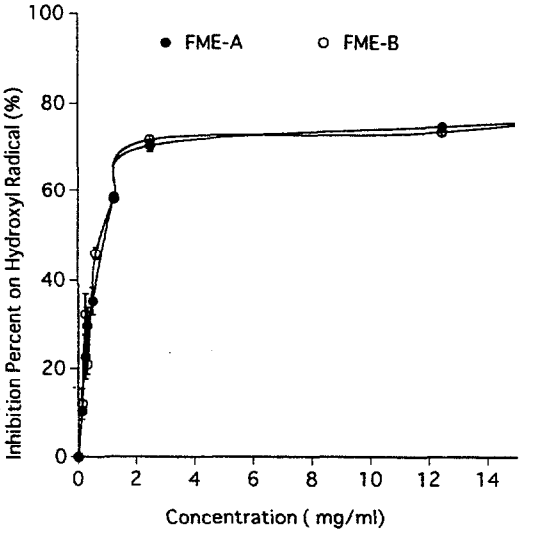

Fig. 3. Scavenging effect of FME-A and FME-B on hydroxyl radicals (•OH) generated by Fenton reagent. Each bar represents the mean ± SD of 3 or 4 determinations.

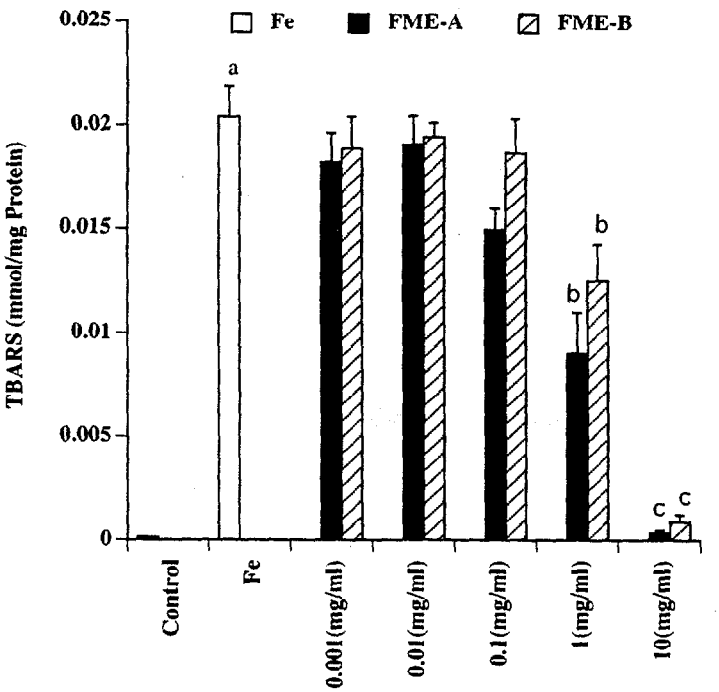

Fig. 4. Inhibitory effect of FME-A and FME-B on thiobarbituric acid reactive substances formation in rat cortex homogenates induced by Fe^{2+}-ascorbic acid. Each bar represents the mean ± SD of 5 determinations. a $p<0.001$ vs control; b $p<0.05$, c $p<0.001$ vs Fe.

In control (untreated) experiments, the amount of TBARS formed in rat cortex homogenates was 0.1 nmol/mg protein. Lipid peroxidation in rat cortex homogenates was stimulated by the addition of Fe^{2+}-ascorbic acid. FMEs inhibited Fe^{2+}-ascorbic acid-induced TBARS formation in a concentration—dependent manner (Fig.4). At a concentration of 1mg/ml, FME-A could inhibit TBARS formation by 55.88%, and FME-B by 38.73%. At a concentration of 10mg/ml, FME-A could inhibit TBARS formation by 98.04%, and FME-B by 95.59%.

The effects of FMEs on lipid peroxidation in $FeCl_3$-injected isocortexes of rats were shown in Fig.5-7. The TBARS, MDA and 4-HNE levels increased significantly following iron ion injection into the cortex, compared to saline injection. Both FME-A and FME-B inhibited lipid peroxidation induced by ferric ion in the cortex, as indicated by TBARS assay and MDA assay (Fig. 5 and 6). The 4-HNE level in the cortex was reduced significantly only by FME-B at higher dose (Fig.7).

DISCUSSION

Brain homogenate is useful for investigation of lipid peroxidation and has also been used as a preparation to elucidate "antioxidant activity"(26,27). In vitro experiments showed that ferrous ion initiated lipid peroxidation in cortex homogenates and FMEs could inhibit lipid peroxidation, as indicated by TBARS formation.

One animal model of a neurological disorder related to free radical production and lipid peroxidation, which has been well established and studied since the report of Willmore (25), is the induction of chronic, recurrent seizure in the albino rat by the intracortical injection of an iron salt solution. Previous studies showed that iron-induced epileptogenesis is related to the production of hydroxyl radical (28) and superoxide radicals (29). TBARS formation in the model indicated lipid peroxidation occurred in the brain. Some natural antioxidants have been found to protect $FeCl_3$ induced epileptic seizure of rats (30-33). Thiobarbituric-acid method is a classic determination of study of the level of lipid peroxidation. Because it is affected by much interference its

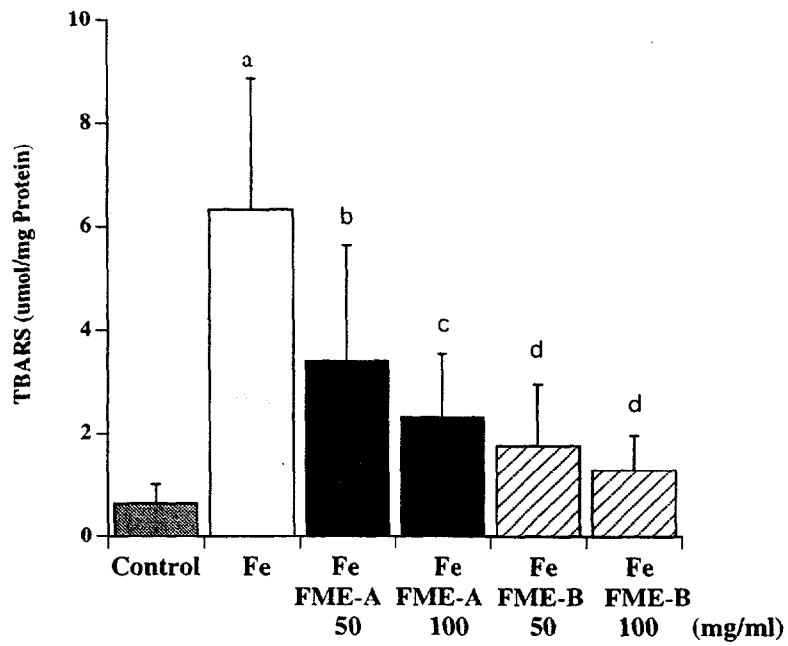

Fig. 5. Effect of FME-A and FME-B on thiobarbituric acid reactive substances formation in the iron-induced epileptic foci of rats. The results are expressed as means ± SD of 7 determinations. a $p<0.001$ vs control; b $p<0.05$, c $p<0.005$, d $p<0.001$ vs Fe.

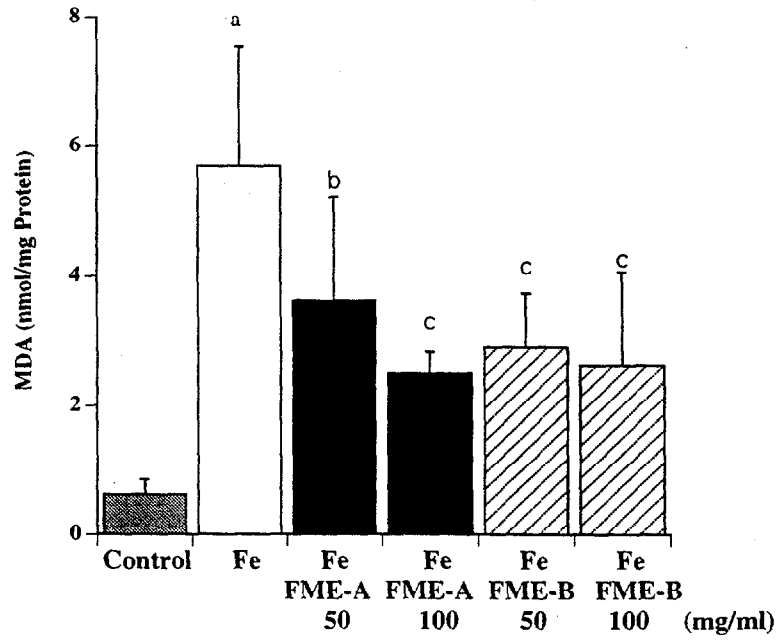

Fig. 6. Effect of FME-A and FME-B on malonaldehyde formation in the iron-induced epileptic foci of rats. The results are expressed as mean ± SD of 7 determenations. a $p<0.001$ vs control; b $p<0.05$, c $p<0.005$ vs Fe.

reproducibility is not satisfactory (34). Malonaldehyde and 4-hydroxyalkenals, such as 4-hydroxy-2(E)-nonenal (4-HNE), are important decomposition products of peroxides derived from polyunsaturated fatty acids and related esters. It has been recognized that the measurement of such aldehydes provides a convenient index of lipid peroxidation (35). Our results indicated that Fe(III) induced lipid peroxidation in the rat cortex shown by the increased production of TBARS, MDA and 4-HNE. FME-A and FME-B both inhibited lipid peroxidation induced by Fe(III) in cortex as expressed by TBARS and MDA formation, although the 4-HNE level in the cortex was reduced significantly only by FME-B at higher dose. Ferrous ion stimulates lipid peroxidation through various mechanisms, e.g. the decomposition of lipid peroxides, the generation of hydroxyl radicals or the formation of perferryl or ferryl species. Though the mechanism by which ferric ion induces lipid peroxidation in the cortex needs further investigation, FMEs were effective in inhibiting the ferrous ion-stimulated lipid peroxidation of rat cortex homogenate and ferric ion-induced lipid peroxidation in the cortex. This could be because FMEs 1) form complexes with

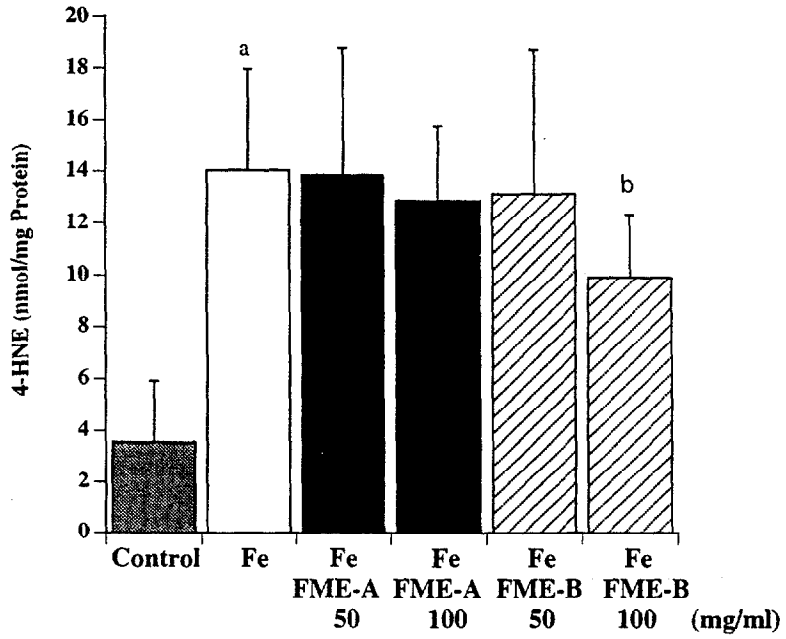

Fig. 7. Effect of FME-A and FME-B on 4-hydroxynonenal formation in the iron-induced epileptic foci of rats. The results are expressed as mean ± SD of 7 determinations. a $p<0.001$ vs control; b $p<0.05$ vs Fe.

ferrous ions, and/or 2) scavenge free radicals. The latter concept is further supported by the DPPH test that showed the high reactivity of FMEs with lipid-soluble free radical.

The ability of FMEs to function as antioxidants has been verified in this study. Experimental results indicate that FMEs are scavengers of the stable-lipid soluble free radical DPPH, of hydroxyl radical, and of superoxide radical. They inhibited lipid peroxidation not only in vitro, but also in vivo. The in vivo result indicated that components of FMEs could pass the blood-brain barrier. The results also showed that there is no significant difference between the scavenging activities of FME-A and FME-B, which are not significantly different in composition. These results suggest that one of the mechanisms of the anti-inflammatory effect of Fructus Momordicae may be contributed by this antioxidant action and FMEs may be an effective antioxidant medicine for brain diseases related to free radical injury. The results do not allow us distinguish which component of the FMEs is responsible for the antioxidant properties. The ability to scavenging free radicals could be due to a particular component, as well as to the interactions of different

chemicals, as in the case with EGb 761 (36). FMEs , which are a natural product, appear to be promising antioxidant and may be useful in treating a wide range of free radical-induced diseases.

Acknowledgments

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11

Less Common High-Potency Sweeteners

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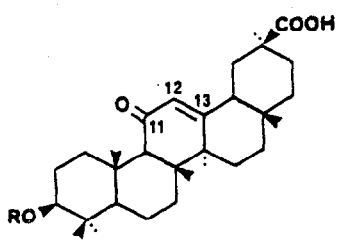
INTRODUCTION

In this chapter, recent progress on the study and utilization of several less commonly encountered naturally occurring and synthetic sweet substances will be described. Several of the natural sweeteners discussed have served as template molecules for extensive synthetic modification. Greater emphasis will be provided for those compounds that have commercial use as high-potency sweeteners or as flavoring agents in one or more countries, and priority will also be given to those sweet compounds for which there is recent published information. Following the format of the earlier version of this chapter (1), substances that modify the sweet taste response will also be mentioned.

NATURALLY OCCURRING COMPOUNDS

Glycyrrhizin

Glycyrrhizin, which is also known as glycyrrhizic acid, is an oleanane-type triterpene glycoside whose use as a sweetener has been covered in several recent reviews (1–4). This compound, a diglucuronide of the aglycone, glycyrrhetinic acid (Fig. 1), is extracted from the roots of licorice (*Glycyrrhiza glabra* L., Fabaceae) and other species in the genus *Glycyrrhiza*. In its natural form, the compound occurs in the plant in yields of 6–



	R
Glycyrrhizin	β-glcA^2-β-glcA
Apioglycyrrhizin	β-glcA^2-β-api

Figure 1 Structures of glycyrrhizin and apioglycyrrhizin. (GlcA = S-glucuronopyranosyl; api = S-apiofuranosyl.)

14% w/w as a mixture of various metallic salts. Well-established procedures are available for the extraction and purification of glycyrrhizin from the plant. Conversion of glycyrrhizin to ammoniated glycyrrhizin, the fully ammoniated salt of glycyrrhizin, results in a more water-soluble compound that is reasonably stable at elevated temperatures. Glycyrrhizin has been rated as approximately 50–100 times sweeter than sucrose, although it has a slow onset of sweet taste and a long aftertaste. Ammoniated glycyrrhizin has similar hedonic properties to glycyrrhizin. Its sweetness intensity, which is about 50 times sweeter than sucrose, is increased in the presence of sucrose (1–4). Attempts to improve the sensory parameters of glycyrrhizin by synthetically modifying its carbohydrate moieties have not so far led to any sweeter or more pleasant-tasting analoges (2,3). However, apioglycyrrhizin (Fig. 1), a glycyrrhizin analog recently isolated from the roots of *Glycyrrhiza inflata* Batal, has been found to be about twice as sweet as the parent compound (5). Periandrins I–IV are additional naturally occurring oleanane-type triterpene glycosides that were isolated by Hashimoto and colleagues from the roots of *Periandra dulcis* L. (Fabaceae) (Brazilian licorice). These compounds are 90–100 times sweeter than sucrose, but occur in the plant in low yields and are somewhat difficult to purify from bitter substances with which they co-occur (2,4).

Glycyrrhiza root extracts that are constituted by at least 90% w/w pure glycyrrhizin, are widely used in Japan for sweetening and flavoring foods, beverages, medicines, cosmetics, and tobacco (1,6,7). In 1987, extracts of *Glycyrrhiza* species containing glycyrrhizin were estimated to have a share of the Japanese high-potency sweetener market (27%) that

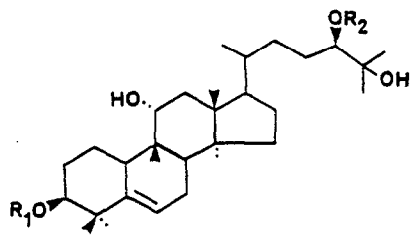
was second only to *Stevia rebaudiana* sweeteners, with a sales volume of 2 billion yen (6). Roots of *Glycyrrhiza* species for the Japanese market are produced mainly in the People's Republic of China, the Soviet Union, Afghanistan, and Pakistan. In addition, extracts of *Glycrrhiza* roots are imported into Japan from the United States and the People's Republic of China (6). Ammoniated glycyrrhizin is included in the GRAS (Generally Recognized as Safe) list of approved natural flavoring agents by the U.S. Food and Drug Administration. There are many applications of this compound as a flavorant, flavor modifier, and foaming agent. While it is very useful for incorporation into confectionery and dessert items, ammoniated glycyrrhizin is only employed in carbonated beverages that do not have too low a pH, since this substance tends to precipitate at pH levels below 4.5 (1).

Glycyrrhizin exhibits a number of other biological activities in addition to its sweetness, as exemplified by its antiallergic, antiinflammatory, antitussive, and expectorant actions. Unfortunately, the widespread use of glycyrrhizin and ammoniated glycyrrhizin by humans has been shown to lead to pseudoaldosteronism, which is manifested by hypertension, edema, sodium retention and mild potassium diuresis (2,7,8). The 11-oxo-$\Delta^{12,13}$- functionality in ring C of the aglycone of glycyrrhizin has been attributed as the part of the molecule responsible for this untoward activity (7,9). The Ministry of Health in Japan has issued a caution stipulating that glycyrrhizin should be limited to less than 200 mg/day when used in drug formulations (8). Glycyrrhizin, at a level of 0.5–1%, has been shown to inhibit in vitro plaque formation mediated by *Streptococcus mutans*, a cariogenic bacterial species. As a consequence, it has been suggested that glycyrrhizin is suitable for wider use as a vehicle and sweetener for medications employed in the oral cavity (9).

Mogrosides

Mogrosides IV and V are sweet curcurbitane-type triterpene glycoside constituents of the dried fruits of the Chinese plant *lo han kuo*. This species, a member of the family Cucurbitaceae, was accorded the binomial *Momordica grosvenorii* Swingle in 1941, which was then changed to *Thladiantha grosvenorii* (Swingle) C. Jeffrey in 1979. However, new evidence has indicated that the name of this plant should be *Siraitia grosvenorii* (Swingle) C. Jeffrey. Chemical studies on the sweet principles of this plant did not begin until the 1970s (2,4).

The structures of mogrosides IV and V (Fig. 2) were established by Takemoto and colleagues after extensive chemical and spectroscopic studies (10–12). The major sweet principle of *lo han kuo* fruits is mogro-



	R_1	R_2
Mogroside IV	β-glc^6-β-glc	β-glc^2-β-glc
Mogroside V	β-glc^6-β-glc	β-glc^2-β-glc (β-glc^6)

Figure 2 Structures of mogroside IV and mogroside V. (Glc = S-glucopyranosyl.)

side V, which occurs in concentration levels of over 1% w/w (2). Mogroside V is a polar compound, since it contains five glucose residues, which readily permit its extraction into either water or 50% ethanol. Aqueous solutions containing mogroside V are stable when boiled (1). Mogroside V was rated as being 256 times sweeter than sucrose by a human taste panel, when a 0.02% w/v solution was tested (10). However, this compound has been found to possess a lingering, licorice-like aftertaste (1,2). Three other sweet cucurbitane glycosides have been isolated from two species in the genus *Hemsleya*, although none of these compounds was ranked as more than 80 times sweeter than sucrose, when tested at a concentration of 0.025% w/w (13,14).

Extracts made from *lo han kuo* fruits have long been used by local populations in Kwangsi province in southern China for the treatment of colds, sore throats, and minor stomach and intestinal complaints (2). Preparations made from *lo han kuo* are available in Chinese medicinal herb stores in several countries including the United States. It was estimated that the domestic demand of *lo han kuo* fruits containing mogroside V for food and medicinal products in Japan in 1987 was two metric tons, representing a sales volume of 40 million yen (6).

Safety studies on mogroside V have not been extensive to date. The compound has been found to be nonmutagenic, when tested in a forward mutation azaguanine assay using *Salmonella typhimurium* strain

TM677 (2,4). Mogroside V produced no mortalities when administered by oral intubation at doses up to 2 g/kg body weight in acute toxicity studies in mice, and an aqueous extract of *lo han kuo* fruits exhibited an LD_{50} in mice of > 10 g/kg body weight (2,4). There appear to have been no adverse reactions among human populations who have ingested aqueous extracts of *lo han kuo* fruits, which would be expected to contain substantial quantities of mogroside V. Therefore, *lo han kuo* extracts containing mogroside V might well be worthy of wider application for sweetening purposes in the future, because of their apparent safety, in addition to favorable sensory, stability, solubility, and economic aspects.

Phyllodulcin

Phyllodulcin is produced from its naturally occurring glycoside form by enzymatic hydrolysis when the leaves of *Hydrangea macrophylla* Seringe var. *thunbergii* (Siebold) Makino (Saxifragaceae) and other species in this genus are crushed or fermented. Phyllodulcin (Fig. 3) is a dihydroisocoumarin and was initially isolated in 1916 and structurally characterized in the 1920s. In 1959, this sweet compound was found to have 3*R* stereochemistry (1–4). Phyllodulcin has been detected in the leaves of *H. macrophylla* subsp. *serrata* var. *thunbergii* in levels as high as 2.36% w/w (2). Several patented methods are available for the purification of phyllodulcin. In one such procedure, after initial extraction from the plant with methanol or ethanol, hydragenol (a nonsweet analog of phyllodulcin) and pigment impurities were removed after pH manipulations and extraction with chloroform. Phyllodulcin was then selectively extracted in high purity at pH 10 with a non-polar solvent (15). The relative sweetness of phyllodulcin has been variously reported as 400 and 600–800 times sweeter than sucrose, although the compound exhibits a delay in sweetness onset and a licorice-like aftertaste (16). There have been extensive attempts to modify the phyllodulcin structure in order to produce compounds with improved sensory characteristics, which have recently been subjected to detailed review (3). As a result of such investigations, it has been established that the 3-hydroxy-4-methoxyphenyl unit of phyllodul-

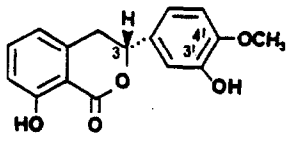


Figure 3 Structure of phyllodulcin.

cin must be present for the exhibition of a sweet taste, but the phenolic hydroxy group and the lactone function can be removed without losing sweetness (3,4,16). To date, the phyllodulcin derivatives that have been produced synthetically seem to have limitations in terms of their water solubility, stability, and/or sensory characteristics (3,4,16).

The fermented leaves of *H. macrophylla* var. *thunbergii* ("Amacha") are used in Japan to produce a sweet tea that is consumed at *Hamatsuri*, a Buddhist religious festival (1,2). A 1987 estimate indicated that the demand for extracts of *Hydrangea* species containing phyllodulcin was 1 metric ton, with a value of 15 million yen (6). Pure phyllodulcin has been found to be nonmutagenic in a forward mutation assay, and also not acutely toxic for mice, when administered by oral intubation at up to 2 g/kg body weight (2). The low solubility in water and the sensory limitations of phyllodulcin that have been referred to would seem to limit the prospects of this compound being more widely utilized as a sweetening agent in the future.

Sweet Proteins

Thaumatin

Thaumatins I and II are the major sweet proteins that have been obtained from the arils of the fruits of the West African plant *Thaumatococcus daniellii* (Bennett) Benth. (Marantaceae). Thaumatin I has a relative sweetness intensity of between 1600 and 3000, when compared with sucrose on a weight basis. Thaumatin protein (which is known by the trade-name of Talin® protein) was comprehensively reviewed by Higginbotham in the first edition of *Alternative Sweeteners* (17), in terms of botany, production, biochemistry, physical characteristics, sensory parameters, sweetness synergy with other substances, applications (including flavor potentiation and aroma enhancement effects), safety assessment, cariogenic evaluation, and regulatory status. Since a further detailed review of thaumatin by van der Wel has also appeared in the literature (18), this subject is not covered in depth in the present chapter.

Talin® protein was initially permitted as a natural food additive in Japan in 1979 (17). In 1987, there was an estimated Japanese demand of 200 kg of thaumatin, which was valued at a price of 350,000 yen per kilogram (6). Despite the fact that Talin® protein has been approved as a sweetener in the United Kingdom and Australia and is being similarly reviewed in several other countries (17), it now appears that the major future use of this product will be as a flavor enhancer. In the United States, Talin® protein was accorded GRAS status as a flavor adjunct for chewing gum in 1984 (17).

Monellin

The sweet protein isolated from the fruits of another African plant, *Dioscoreophyllum cumminsii* (Stapf) Diels (Menispermaceae), has been called "monellin," a substance composed of two polypetide chains, of molecular weight 11,000 daltons (1). "Monellin" is actually monellin 4, one of five sweet proteins, named monellins 1–5, that have been isolated from this plant. Present procedures, in which buffered aqueous extracts of *D. cumminsii* fruit pulps are fractionated, enable 3–5 g of protein to be purified per kg of fruit, with a sweetness relative to 7% w/v sucrose of 1500–2000 times (18). Monellin is costly to produce, and its plant of origin is difficult to propagate. In addition, the compound has a slow onset of taste, along with a persistent aftertaste, and its sweet effect is both thermolabile and pH sensitive (1,2,18). No toxicological data are yet available for monellin (18). Therefore, unless new formulations can be produced to limit these disadvantages, it looks as if monellin will not be developed in the near future as a high-intensity sweetener and will have greater utilization as a molecular tool for the laboratory investigation of the sweetness sensation (1,2,18).

Other Sweet Proteins

Mabinlins I and II are sweet proteins that are produced in the seeds of *Capparis masaikai* Lévl (Capparidaceae), a plant which grows in Yunnan province in the People's Republic of China. The molecular weights of mabinlins I and II are 11,600 and 10,400 daltons, respectively, and both proteins are composed of single polypeptide chains, and have some 80 amino acid residues in common. These sweet albumins were found to occur in a combined yield of 13% w/w of the dry weight of the defatted seeds, with mabinlin I being the major sweet component. Mabinlins I and II were treated with 50% aqueous acetone, and then purified by chromatography over cellulose. The compounds are reported to be less sweet than thaumatin and monellin, although similar in sweetness quality. The sweet taste of mabinlin II persists on incubation at 80°C for 48 hours at pH 6, although the sweetness of the less stable mabinlin I is lost after 30 minutes when stored under the same conditions. Children are reported to chew the seed meal of *C. masaikai* because of its sweet taste and because it imparts a sweet taste to water drunk later. No pharmacological studies have been performed on either the mabinlins or on *C. masaikai* seed meal, although the latter is used in traditional Chinese medicine to treat sore throats and as an antifertility agent (19).

Pentadin is a sweet protein that was recently reported as a constituent of the fruits of the African plant *Pentadiplandra brazzeana* Baillon (Pentadi-

plandraceae). The compound has an estimated molecular weight of 12,000 daltons and was obtained from the plant by water extraction, ultrafiltration, and gel filtration. Pentadin has been estimated to have a sweetness potency about 500 times that of sucrose on a weight basis. Using a rhesus monkey, 0.1% pentadin gave a steeper onset and more rapid stimulatory-response decline than did 0.02% thaumatin and monellin. Further studies are planned to more thoroughly characterize pentadin (20).

Miscellaneous Highly Sweet Plant Constituents

Hernandulcin

Hernandulcin (Fig. 4) is a bisabolane sesquiterpene that was isolated as a minor constituent in a petroleum ether-soluble extract of the aerial parts of the herb *Lippia dulcis* Trev. (Verbenaceae), collected in Mexico. This plant was known to be sweet by the Aztec people, according to the Spanish physician Francisco Hernández, who wrote a monograph entitled "Natural History of New Spain" between 1570 and 1576. The *L. dulcis* sweet constituent was named in honor of Hernández, and was rated as 1000 times sweeter than sucrose on a molar basis when assessed by a taste panel (21). Racemic hernandulcin was synthesized by a directed aldol condensation from two commercially available ketones (21,22), and the naturally occurring (6*S*,1′*S*)-diastereomer of this compound was produced in the laboratory from (*R*)-limonene (23). When the functional groups of hernandulcin were modified, no sweet compounds resulted (24). However, this investigation enabled the conclusion to be made that, in addition to the C-1′ hydroxyl and the C-1 carbonyl groups of hernandulcin, which represent the AH and B groups in the Shallenberger model of sweetness, the C-4′–C-5′ double bond appears to be a third functionality necessary for the exhibition of a sweet taste by this compound (22,24). Racemic hernandulcin was not mutagenic and not acutely toxic for mice at the doses tested in preliminary safety studies (21,22). Unfortunately, the high sweetness potency of hernandulcin is marred by an unpleasant aftertaste and a somewhat bitter-tasting nature (21).

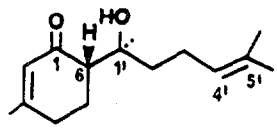
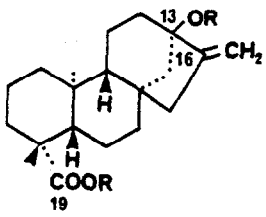


Figure 4 Structure of hernandulcin.



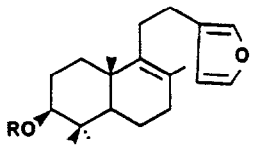
	R
Rubusoside	β-glc

Figure 5 Structure of rubusoside. (Glc = S-glucopyranosyl.)

Rubusoside

Tanaka and co-workers have determined the *ent*-kaurene diterpene rubusoside (Fig. 5) to be responsible for the sweet taste of the leaves of *Rubus suavissimus* S. Lee (Rosaceae), which is indigenous to southern regions of the People's Republic of China (2,4). Rubusoside is extractable from the plant with hot methanol and occurs in high yield in the leaves of *R. suavissimus* (> 5% w/w) and at lower concentration levels in the fruits of this plant (25). When evaluated at a concentration level of 0.025%, rubusoside was rated as possessing 114 times the sweetness of sucrose, although its quality of taste sensation was marrred by some bitterness (2,4). Several analogs of rubusoside have been produced by treatment with the cyclodextrin glucosyltransferase produced by *Bacillus megaterium* strain No. 5, and some of these products proved to be sweeter and more pleasant-tasting than rubusoside itself (26).

A sweet tea called *Tian-cha*, prepared from the leaves of *R. suavissimus*, is consumed as a summer beverage in the Guangxi Autonomous Region of the People's Republic of China. Also, during festivals, local populations mix aqueous extracts of this plant with rice in order to make cakes. In recent years, teas made from the leaves of *R. suavissimus* have been used in folk medicine to treat diabetes, hypertension, and obesity. In an acute toxicity test on rubusoside, the LD_{50} was established as about 2.4 g/kg body weight, when administered orally to mice. Subsequently, in a subacute toxicity study, rubusoside was incorporated into the diet of mice for 60 days at a dose of one-tenth of its LD_{50}, and no distinct toxicity nor side effects were observed (27). However, the aglycone of rubusoside is steviol (*ent*-13-hydroxykaur-16-en-19-oic acid), which has been shown to be mutagenic in a bacterial forward mutation assay when



	R
Baiyunoside	β-glc^2-β-xyl
Phlomisoside I	β-glc^2-α-rha

Figure 6 Structures of baiyunoside and phlomisoside I. (Glc = S-glucopyranosyl; xyl = S-xylopyranosyl; rha = S-rhamnopyranosyl.)

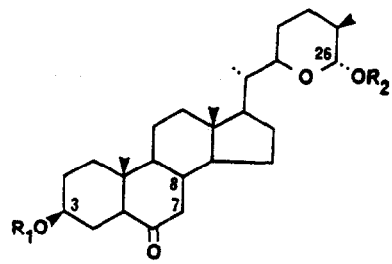
metabolically activated (28). The mutagenicity of steviol is discussed more fully in Chapter 9.

Baiyunoside

Baiyunoside (Fig. 6) is a labdane-type diterpene glycoside, based on the aglycone (+)-baiyunol, that was first isolated in 1983 by Tanaka and co-workers from a plant used in Chinese medicine, namely, *Phlomis betonicoides* Diels (Labiatae) (2,4,29). This butanol-soluble compound was found to be about 500-fold sweeter than sucrose and to possess a lingering aftertaste lasting more than one hour. Phlomisoside I (Fig. 6) is a further sweet constituent of *P. betonicoides*, although its sensory characteristics have not been detailed (29). Synthetic routes are available for both (±)- and (+)-baiynunol (30,31). A general glucosylation procedure has been developed for baiyunol and has not only been used to produce baiyunoside synthetically, but offers a general method for the preparation of baiyunoside glycosidic analogs, in the hope that one or more might exhibit better sensory properties than the parent compound (32). No safety studies appear to have been performed thus far on baiyunoside.

Steroidal Saponins

Osladin (Fig. 7) is a steroid saponin constituent of the fern *Polypodium vulgare* L. (Polypodiaceae), which was isolated and structurally characterized by Herout and co-workers in 1971 (1–4). The stereochemistry of the aglycone of osladin was established by Havel and Cerny in 1975, although the configuration of the S-rhamnopyranosyl moiety at C-26 of this glycoside has not yet been determined (2,4). While osladin has been rated as 3000 times as sweet as sucrose, its hedonic taste characteristics are not described in the literature.



	R_1	R_2	Other
Osladin	β-glc^2-α-rha	rha	--
Polypodoside A	β-glc^2-α-rha	α-rha	$\Delta^{7,8}$
Polypodoside B	β-glc	α-rha	$\Delta^{7,8}$

Figure 7 Structures of some sweet steroidal saponins. (Glc = D-glucopyranosyl; rha = L-rhamnopyranosyl.)

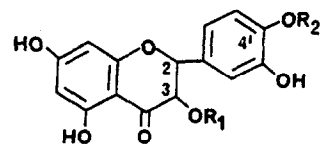
In recent work, two sweet compounds, polypodosides A and B (Fig. 7), which are closely related in structure to osladin, have been isolated from the rhizomes of *Polypodium glycyrrhiza* D.C. Eaton (Polypodiaceae) 33,34). This plant, known by the common name of "Licorice fern," is a North American species that is native to the Pacific northwest. Polypodoside A, the major sweet constituent of *P. glycyrrhiza*, has a neohesperidose (2-*O*-α-S-rhamnopyranosyl-β-S-glucopyranose) unit attached to C-3 of the aglycone, as well as a C-26 affixed S-rhamnopyranosyl unit (with a β configuration). The aglycone of polypodoside A (polypodogenin) corresponds to the $\Delta^{7,8}$-derivative of the aglycone of osladin. Polypodoside A was found to be nonmutagenic and not acutely toxic for mice, when dosed by oral intubation at up to 2 g/kg body weight. In subsequent sensory tests using a small human taste panel, polypodoside A was assessed as having 600 times the sweetness intensity of a 6% w/v sucrose solution, but also revealed a licorice-like off-taste and a lingering aftertaste (33). Polypodoside B, an isolate similar structurally to polypodoside A, except that it has a glucose unit affixed to C-3, was found to be only slightly sweet (34). Comparison of polypodosides A and B with other compounds based on the same aglycone has indicated that polypodogenin glycosides must be bisdesmosidic in order to exhibit a sweet taste (34). The potential of polypodoside A for commercialization is marred by its relative insolubility in water, its sensory characteristics, and difficulties in collecting *P. glycyrrhiza* rhizomes (33). It is probable that osladin

will suffer from similar limitations, in addition to its extremely low concentration levels in its plant of origin, as has already been noted (1).

Dihydroflavonol Sweeteners

Naturally occurring flavonoids are generally regarded as either bitter- or neutral-tasting compounds, although a number of sweet or bittersweet dihydrochalcone glycosides have been reported, including glycyphyllin, phloridzin, and trilobatin (2,4). Recently, a new class of sweet flavonoids was discovered, namely, the dihydroflavonol sweeteners. In an initial report, the sweetness and astringency of the stembark of *Glycoxylon huberi* Ducke (Sapotaceae) was correlated with the presence of four dihydroflavonol constituents, although the individual compounds were not assessed for the presence or absence of sweetness (35).

The known compound dihydroquercetin 3-acetate (Fig. 8) was isolated as a sweet constituent of the young shoots of *Tessaria dodoneifolia* (Hook. & Arn.) Cabrera (Asteraceae). Dihydroquercetin 3-acetate-4′-methyl ether (Fig. 8), a synthetic derivative of this sweet plant constituent, was determined as possessing about 400 times the sweetness potency of a 3% w/v sucrose solution, and exhibited no bitterness, although it had a somewhat slow onset of sweet taste. By comparison, dihydroquercetin 3-acetate was rated as about 80 times sweeter than 3% w/v sucrose. Both these quercetin derivatives were found to be nonmutagenic and not acutely toxic for mice. The aerial parts of *T. dodoneifolia* are used in Paraguay in folk medicine as an emmenogogue, and the plant is referred to colloquially as *Kaá hê-é* (sweet herb) (36).



	R_1	R_2	
Quercetin 3-acetate	acetyl	H	(2*R*,3*R*)
Quercetin 3-acetate-4′-methyl ether	acetyl	CH_3	
Neoastilbin	α-rha	H	(2*S*,3*S*)

Figure 8 Structures of some sweet dihydroflavonol derivatives. (Rha = S-rhamnopyranosyl.)

Neoastilbin (Fig. 8) is a further dihydroflavonol that has been reported to possess a sweet taste. This compound is a rhamnoside derivative of taxifolin that is produced by the leaves of *Engelhardtia chrysolepis* Hance (Juglandaceae), a species which grows in southern parts of the People's Republic of China. The sweetness intensity of neoastilbin has not been reported. The leaves of *E. chrysolepis* are used as sweet tea by local populations where they are available (37).

SYNTHETIC COMPOUNDS

Oximes

Perillartine (Fig. 9), the *α-syn*-oxime of perillaldehyde, has been known to be highly sweet since 1920, and is reported to be up to 2000 times sweeter than sucrose (3,38,39). In contrast, perillaldehyde itself (Fig. 9), a major constituent of the volatile oil of *Perilla frutescens* (L.) Britton (Labiatae), is only slightly sweet. Perillartine is used commercially in Japan as a replacement for maple syrup or licorice for the sweetening of tobacco, but more widespread use of this compound for sweetening purposes has been restricted by a limited solubility in water, an appreciably bitter taste, as well as a menthol-licorice off-taste that accompanies sweetness (2,3,38,39).

The intense sweetness and structural simplicity of perillartine have promoted the synthesis of numerous analogs (3,38,39). This work has not only led to a better understanding of the functional groups in compounds of the oxime class that confer sweetness and bitterness, but has also led to the development of several improved sweet compounds. One of the most promising of such derivatives is SRI oxime V (Fig. 9). This compound is 450 times sweeter than sucrose on weight basis and exhibits much improved water solubility when compared with perillartine.

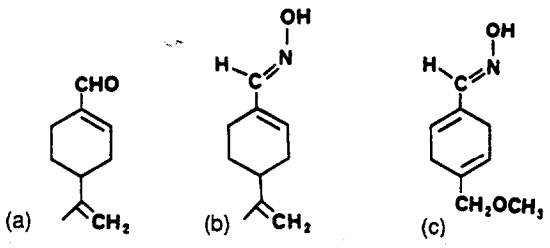


Figure 9 Structures of (a) perillaldehyde, (b) erillartine, and (c) SRI oxime V.

SRI oxime V has no undesirable aftertaste and is stable above pH 3 (1–3,38–40). This substance was shown not to be a bacterial mutagen in the Ames assay, and to exhibit an LD_{50} of > 1 g/kg body weight in the rat after a single oral dose (40). The compound is readily absorbed and metabolized, with excretion nearly quantitative within 48 hours after administration to the rat, dog, and rhesus monkey. The major metabolites of SRI oxime V were found to be products resulting from oxidation of either the methoxymethyl or the aldoxime moieties, as well as those occurring after thioalkylation and glucuronidation (40). Subchronic toxicity tests on this compound conducted in rats with a diet containing 0.6% SRI oxime V for 8 weeks revealed no apparent toxic effects. It has been suggested that SRI oxime V shows such promise as an artificial sweetener that a chronic toxicity test is warranted (40).

Urea Derivatives

Dulcin (*p*-ethoxyphenylurea) has been known to be sweet for over a century. The compound is about 200 times sweeter than sucrose and was briefly marketed as a sucrose substitute in the United States. Commercial use of this compound was discontinued after it was found to be toxic to rats at a low dose. Dulcin has also been found to be mutagenic (1,39).

Another group of sweet ureas of more recent interest are the carboxylate-solubilized *p*-nitrophenyl derivatives, which were discovered by Peterson and Müller (3,39). Suosan, the sodium salt of *N*-(*p*-nitrophenyl)-*N'*-(β-carboxyethyl)-urea (Fig. 10), is representative of this series and has been reported as about 350 times sweeter than sucrose, although it has significant bitterness. Other compounds in this class are even sweeter than suosan (3,39,42). Structure–sweetness relationships have been investigated for the sweet-tasting arylureas (3,42,43).

Miscellaneous Compounds

Tryptophan Derivatives The sweetness of derivatives of the amino acid tryptophan was discovered by Kornfeld and his co-workers in 1968, when it was observed that racemic 6-trifluoromethyltryptophan has an

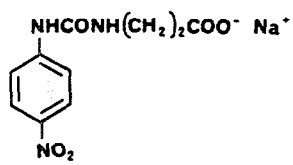


Figure 10 Structure of suosan.

intensely sweet taste (3,44). Additional studies demonstrated that these compounds are sweet when in the S form, with 6-chloro-S-tryptophan being some 1000 times sweeter than sucrose. The S form of this compound is tasteless but has been found to possess antidepressant activity (44).

It was reported by Finley and Friedman that racemic *N'*-formyl and *N'*-acetyl derivatives of kynurenine, an intermediate in the metabolism of tryptophan, are approximately 35 times sweeter than sucrose and elicit an immediate sweet taste on contact with the tongue (38,44,45). More recently, the 6-chloro derivative of kynurenine [3-(4-chloroanthraniloyl)-SS-alanine] (Fig. 11), has been reported to be 80 times sweeter than sucrose and to possess no significant aftertaste or off-flavor (45).

Trihalogenated Benzamides 2,4,6-Tribromo benzamides that are substituted in the C-3 position by a carboxyalkyl or a carboxylalkoxy group are intensely sweet. For example, 3-(3-carbamoyl-2,4,6-tribromophenyl) propionic acid (Fig. 12) was rated as 4000 times sweeter than sucrose, although this compound has a slow onset and a slight lingering aftertaste, as well as some bitterness (3,46). Within this compound class, the intensity of sweet taste depends upon the chain length of the carboxyalkyl or carboxyalkoxy group (3,46). The acute toxicities of several tribromo benzamides have been determined in mice, and the results were comparable with analogous data obtained for saccharin and cyclamate (46).

(Phenylguanidino)-acetic Acid Derivatives Nofre and co-workers have recently reported the synthesis of a series of (phenylguanidino)- and {[1-(phenylamino)ethyl]amino}-acetic acid derivatives, with sweetness intensities between 30 and 200,000 times that of a 2% sucrose solution (47).

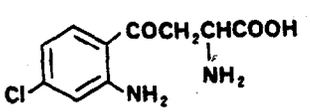


Figure 11 Structure of 3-(4-chloroanthraniloyl)-SS-alanine.

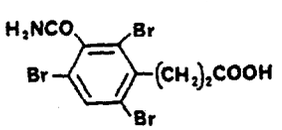


Figure 12 Structure of 3-(3-carbamoyl-2,4,6-tribromophenyl)propionic acid.

These compounds appear to represent some of the most potent sweeteners discovered to date.

SWEETNESS MODIFYING SUBSTANCES

Sweetness Inducers and Enhancers

Miraculin

Miraculin is a basic glycoprotein constituent of the fruits of *Richardella dulcifica* (Schumach. & Thonning) Baehni [formerly *Synsepalum dulcificum* (Schumach. & Thonning) DC.] (Sapotaceae) (miracle fruit), that has the propensity of making sour or acidic materials taste sweet (1,2). Although miracle fruit concentrates are no longer commercially available in the United States as a food additive (1), efforts have continued in the structural characterization of this substance. Miraculin is single polypeptide of 191 amino acid residues, with a carbohydrate content of 13.9% and an overall molecular weight of 24,600 daltons. The complete amino acid sequencing of miraculin was recently carried out by Kurihara and coworkers (48).

Other Sweetness Enhancers

A number of plant constituents have sweetness inducing properties, including cynarin, chlorogenic acid, and caffeic acid (2). Arabinogalactin (larch gum) is capable of enhancing the sweetness potency and taste qualities of saccharin, cyclamate, and protein sweeteners such as thaumatin and monellin (1).

Sweetness Inhibitors

2-(4-Methoxyphenoxy)propanoic Acid

A series of aralkylcarboxylic acid salts, inclusive of the sodium salt of 2-(4-methoxyphenoxy)propanoic acid (Fig. 13) have been found to in-

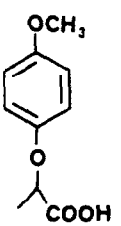


Figure 13 Structure of 2-(4-methoxyphenoxy)propanoic acid.

hibit the sweetness of sucrose (49). Following the analysis of roasted Colombian Arabica coffee beans by gas chromatography/mass spectrometry and high-performance liquid chromatography, 2-(4-methoxyphenoxy)propanoic acid was found to be a naturally occurring constituent (50). This compound (as its sodium salt) is now commercially available as a flavoring agent intended to modulate high sweetness, under the tradename of Cypha®, and has been accorded Generally Recognized as Safe (GRAS) status, for use up to levels of 150 ppm (51).

Arylalkylketones

Several arylalkylketones and arylcycloalkylketones have also been discovered to inhibit the sweet taste of sucrose as well as other bulk and intense sweeteners. One such compound, the commercially available 3-(4-methoxybenzoyl)propionic acid (Fig. 14), is capable of reducing the sweetness intensity of 40% w/v aqueous sucrose by over a sixfold margin, when present at a 2% w/v concentration at pH 7, compared with when absent from the formulation. This compound and its analogs are recommended for use in soft freeze puddings, infused vegetables, and other food products (52).

Triterpene Glycoside Sweetness Inhibitors

Considerable recent progress has been made in the characterization of plant-derived triterpene glycoside sweetness inhibitors. Gymnemic acid has been known for some time to be a mixture of variously acylated glucuronide derivatives of the oleanane-type aglycone, gymnemagenin, found in the leaves of *Gymnema sylvestre* R. Br. (Asclepiadaceae) (2,4). Two groups have now purified six discrete sweetness inhibitors (Fig. 15) and several inactive analogs from this plant source (53–55), which should be of great utility in future research on the physiology of sweetness. The most abundant antisweetness factor found was gymnemic acid II, and it has been found that the more highly acylated substances are more potent as sweetness inhibitors (53–55).

While the suppressive effect of gymnemic acid affects taste sensations other than sweetness, ziziphin, a constituent of *Ziziphus jujuba* P. Miller (Rhamnaceae), has a selective inhibitory action on sweet taste. Ziziphin (Fig. 16) has been isolated and fully characterized by Kurihara

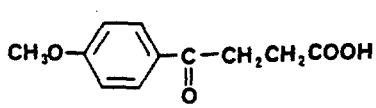
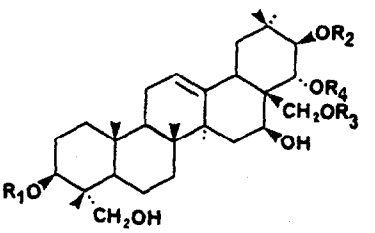
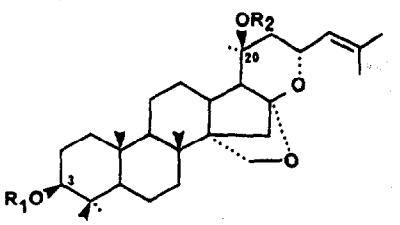


Figure 14 Structure of 3-(4-methoxybenzoyl)propionic acid.



	R_1	R_2	R_3	R_4
Gymnemic acid I	glcA	tiglyl	acetyl	H
Gymnemic acid II	glcA	2-methylbutyryl	acetyl	H
Gymnemic acid III	glcA	2-methylbutyryl	H	H
Gymnemic acid IV	glcA	tiglyl	H	H
Gymnemic acid V	glcA	tiglyl	H	tiglyl
Gymnemic acid VI	glc^3-glcA	tiglyl	H	H

Figure 15 Structures of gymnemic acids I–VI. (GlcA = S-glucuronopyranosyl; glc = S-glucopyranosyl.)



	R_1	R_2
Zizyphin	α-rha^4-α-ara	α-rha-2-Ac-3-Ac

Figure 16 Structure of ziziphin. (Rha = S-rhamnopyranosyl; ara = S-arabinopyranosyl; rha-2-Ac-3-Ac = (2,3-di-O-acetyl)-α-S-rhamnopyranosyl.)

and colleagues and is apparently the only constituent of the plant to manifest a sweetness inhibitory effect (56). Hodulcin, from the leaves of *Hovenia dulcis* Thunb. (Rhamnaceae), which is as yet incompletely characterized, is a further triterpene glycoside that has been found to possess a selective inhibitory effect on sweet taste. However, it has been been established that hodulcin is based on a different aglycone than either gymnemic acids I–VI or ziziphin (57).

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PLANT-DERIVED SWEETENING AGENTS: SACCHARIDE AND POLYOL CONSTITUENTS OF SOME SWEET-TASTING PLANTS*

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Summary

Samples of the sweet-tasting species *Acanthospermum hispidum* DC. (Compositae) (aerial parts), *Boscia salicifolia* Oliv. (Capparidaceae) (stem bark), *Hovenia dulcis* Thunb. (Rhamnaceae) (peduncles) and *Inga spectabilis* Willd. (Leguminosae) (arils) were acquired as part of a continuing search for high-intensity natural sweeteners of plant origin. Following their preliminary safety evaluation, the sweetness of these plants was traced to large amounts of sugars and polyols by taste-guided fractionation, which were identified and quantified using gas chromatography/mass spectrometry. The combined yields of sugars and polyols in the *A. hispidum*, *B. salicifolia*, *H. dulcis*, and *I. spectabilis* samples investigated were 6.9, 10.1, 18.4 and 12.1% w/w, respectively. These yields are much higher than the total saccharide and polyol content (2.4% w/w) of the sweet dried fruits of *Thladiantha grosvenorii* (Swingle) C. Jeffrey (Cucurbitaceae), a species which has previously been reported to contain more than 1% w/w of the intensely sweet triterpene, mogroside V. The dried leaves of *Symplocos tinctoria* (L.) L'Hérit. (Symplocaceae), which were not appreciably sweet, were found to contain only 2.0% w/w of sugars. The results of this investigation, therefore, suggest that unless the saccharide and/or polyol content of a plant part is well over 5% w/w, then it is unlikely to exhibit an overtly sweet taste, unless an intense sweetener is present.

*Part XX in the series "Potential sweetening agents of plant origin". For part XIX, see Choi et al., 1989.

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Introduction

In a research program directed towards the isolation of non-caloric and non-cariogenic sweet compounds of vegetal origin, candidate sweet-tasting plants for study may be selected on the basis of inquiries from local populations in the field or analysis of available ethnobotanical and botanical literature (Kinghorn and Soejarto, 1986, 1989; Soejarto et al., 1983). Other methods of achieving this purpose are the organoleptic testing of herbarium samples (Soejarto et al., 1982) and the perusal of *Index Kewensis* for plant specific epithets suggestive of a sweet taste (Hussain et al., 1988). The use of these approaches, either alone or in combination, has enabled our group to isolate a number of highly sweet substances from plants, including: stevioside, a diterpene glycoside from *Stevia phlebophylla* A. Gray (Soejarto et al., 1982; Kinghorn et al., 1984); hernandulcin, a sesquiterpene from *Lippia dulcis* Trev. (Compadre et al., 1985, 1986); and dihydroquercetin-3-acetate, a dihydroflavonol from *Tessaria dodoneifolia* (Hook. et Arnott) Cabrera (Nanayakkara et al., 1988).

However, it is now apparent that many plants that taste extremely sweet do so not because of the occurrence of intensely sweet constituents, but rather because of the presence of large amounts of sugars and/or polyols. For a given sweet plant, however, such a conclusion can generally be reached only after considerable laboratory effort, comprising taxonomic authentication, solvent extraction, safety evaluation, activity-guided fractionation and compound identification. In the present investigation, we have examined the saccharide and polyol contents of six plants, five of which are distinctly sweet (including one species known to contain significant amounts of a high potency sweetener) and one of which is not sweet. In this manner, we have determined the concentration levels of sugars and polyols that appear to be necessary to confer a distinctly sweet taste to a plant part. Relevant literature data on the six plants studied in this investigation are summarized in the following paragraphs.

Acanthospermum hispidum DC. (Compositae)

A. hispidum is a herb native to South America, which is now widely distributed in Africa, Central America, Florida, Hawaii, India and the West Indies (Standley, 1928; Watt and Breyer-Brandwijk, 1962; Nash and Williams, 1976; Morton, 1981; Correll and Correll, 1982; Nair et al., 1985). The plant is used as a diuretic, febrifuge, sudorific and as a treatment for gonorrhea in some parts of South America (Uphof, 1968; Morton, 1981), and, in Paraguay, a decoction is used for sore throats. Previous phytochemical investigations on *A. hispidum* have indicated the presence of terpenoid and polyphenolic constituents, with some of the former possessing in vitro antineoplastic activity (Nair et al., 1985; Jakupovic et al., 1986). Uphof (1968) described this plant as bitter and aromatic; however, samples collected by our group in Paraguay tasted moderately sweet.

Boscia salicifolia Oliv. (Capparidaceae)

B. salicifolia is an African tree of the open savannah woodland, up to 13 m

high with willow-like foliage, that commonly occurs on termite hills. It is widespread in tropical Africa from Kenya to Zimbabwe and is also found in Northern Nigeria and Angola (Dalziel, 1948; Hutchinson et al., 1954; Irvine, 1961). Parts of the tree are edible, such as the leaves, which are also used as a forage for livestock, with the starchy roots being consumed in times of food scarcity, and the bark and young wood, which are pounded and used to sweeten soup in Nigeria (Dalziel, 1948; Irvine, 1961). Medicinally, the roots, bark and leaves are used to treat rectal infections, some gynecological disorders and to aid in labor, in addition to their use in relieving fever in cattle (Watt and Breyer-Brandwijk, 1962; Khan et al., 1980; Bullough and Leary, 1982). So far, there has been no report published concerning the chemical constituents of this species. However, alcoholic extracts of *B. salicifolia* bark and leaves were shown to exhibit in vitro antibacterial and antifungal activity (Khan et al., 1980).

Hovenia dulcis Thunb. (Rhamnaceae)

This species is indigenous to the Himalayas, China, Korea and Japan (Bailey, 1943; Research Institute of Botany, 1972). It is an ornamental tree with handsome foliage; being extensively cultivated for its sweet, fleshy, swollen and edible peduncles, which taste like bergamot pear (Hooker, 1875; Chun, 1921; Bailey, 1943). The fruits, borne on the red peduncles, are used medicinally in China as a refrigerant and diuretic, and are reported to contain large quantities of dextrose (D-glucose) (Uphof, 1968; Research Institute of Botany, 1972; Keys, 1976). The seeds are reportedly used in the Japanese folk medicine, while the wood is used in construction and for delicate furniture (Inoue et al, 1978). According to Burkill (1966), the dried sweet peduncles are sold in Chinese pharmacies in the Malay Peninsula, where they are used medicinally. One report has indicated that the peduncles of *H. dulcis* contain high levels of sugars, and that they are eaten and used to make alcoholic drinks including wine (Research Institute of Botany, 1972). Previous phytochemical investigations have revealed the presence of saponins and alkaloids in the root bark (Takai et al., 1973; Inoue et al 1978; Kimura et al., 1981). Hodulcin, a sweetness-suppressing constituent of the leaves, has recently been partially characterized as a triterpene saponin (Segecka and Kennedy, 1987).

Inga spectabilis Willd. (Leguminosae)

I. spectabilis belongs to a rather large genus of trees and shrubs native to tropical America and the West Indies (Standley, 1928; Willis and Airy Shaw, 1980). Its fruits, which contain large seeds surrounded by a fleshy, edible white aril, are sold in the markets of Central America for their sweet taste (Standley, 1928; Uphof, 1968). Decoctions of the leaves, bark and root of *Inga* species have been reported by Garcia-Barriga (1974) to be used in Colombian folk medicine for the treatment of diarrhea, dysentery and rheumatism of the joints. However, in Costa Rica there has been no report of any medicinal use of *Inga* by local populations, and people refrain from consuming the fruit, which is known as "guaba machete", because of perceived adverse gastrointestinal

effects. To date, there appears to have been no literature report on the constituents of *I. spectabilis*, although a methanol-soluble extract of another species of the genus, *I. punctata*, was found to possess cytotoxic activity against KB cells (Kingston and Munjal, 1978).

Symplocos tinctoria (L.) L'Hérit. (Symplocaceae)

S. tinctoria is a deciduous or evergreen small tree or shrub widely distributed throughout the tropical and warm regions of America and Asia (Bailey, 1943; Fernald, 1970). Known as "horse-sugar", it grows in the southern and southeastern parts of the United States, from California to Delaware (Small, 1933; Bailey, 1943; Fernald, 1970). The leaves of this plant are reported to be sweetish and consumed by horses and cattle, while both its leaves and bark are the source of a yellow dye (Small, 1933; Fernald, 1970). The roots, which are bitter and aromatic, are used as a tonic (Uphof, 1968). So far, there has been no published report on the chemical constituents of *S. tinctoria*, although a flavonol glycoside and sweet dihydrochalcone glycosides have been isolated from other members of the genus (Tschesche et al., 1980; Tanaka et al., 1980, 1982).

Thladiantha grosvenorii (Swingle) C. Jeffrey (formerly Momordica grosvenorii Swingle) (Cucurbitaceae)

T. grosvenorii is a vine native to the People's Republic of China, where it is widely cultivated in the Kwangsi province (Swingle, 1941). The dried fruits of this plant, known as "Lo Han Kuo", are described as having an intensely sweet taste and are used in China as a household remedy for colds, sore throats, and for minor stomach and intestinal troubles (Swingle, 1941). The sweetness of this plant has been attributed to the sweet saponins, mogrosides V and VI, in addition to glucose and fructose (Takemoto et al., 1983a–c). A previous investigation on commercially available samples of dried sweet *T. grosvenorii* fruits indicated that the content of mogroside V varies in the range of 0.81–1.29% w/w, depending on the anatomical part of the fruit investigated (Makapugay et al., 1985). Mogroside V has been rated as possessing about 250 times the sweetness intensity of sucrose (Takemoto et al., 1983a). Extracts of the dried fruits of *T. grosvenorii* are used commercially for sweetening purposes in Japan (Anonymous, 1985). No report appears to have been published on the detailed saccharide composition of the fruit.

Materials and methods

Experimental materials

Thin-layer chromatography (TLC) was performed using 0.25 mm-thick, aluminium-backed silica gel plates (E. Merck, Darmstadt, F.R.G.), with column chromatography carried out using silica gel (60–230 mesh) obtained from the same company. All solvents used in the extraction, partition and chromatography were glass-distilled whenever possible. Authentic sugars and polyols were purchased from commercial sources.

Plant material

Samples of the following plants were used in the present study: aerial parts of *A. hispidum* DC. (Asuncion, Paraguay, *Soejarto, Kinghorn and Bordas 6068*, 1987), stem bark of *B. salicifolia* Oliv. (Northern Nigeria, *Daramola s.n.*, 1986), stalks of the fruits of *H. dulcis* Thunb. (South Korea, *Chung s.n.*, 1986), arils of the seeds of *I. spectabilis* Willd. (San José, Costa Rica, *Poveda s.n.*, 1985), leaves of *S. tinctoria* (L.) L'Hérit (Charleston, South Carolina, *Dunbar s.n.*, 1986), and fruits of *T. grosvenorii* (Swingle) C. Jeffery (purchased in Hong Kong and from Chinatown, Chicago, Illinois, *Soejarto s.n.*, 1980). Voucher specimens have been deposited at the John G. Searle Herbarium, Field Museum of Natural History, Chicago.

Solvent extraction and partition

The general procedures used for the preparation of solvent extracts of different polarities are as follows. Plant materials were milled and thereafter extracted three times overnight with 80% methanol at room temperature. These extracts were bulked and solvent was evaporated to dryness in vacuo at a temperature not exceeding 40 °C. Each initial 80% methanol extract was dissolved in 75% methanol and partitioned into aliquots of petroleum ether, ethyl acetate and 1-butanol, to afford, in turn, petroleum ether- , ethyl acetate-, butanol- and aqueous methanol-soluble residues on drying. The initial 80% methanol extract and one or more of the other solvent extracts obtained from each species were subjected to acute toxicity and mutagenicity evaluation before being assessed for sweetness by tasting.

A. hispidum (223 g) was extracted with 80% methanol (3 × 500 ml), to give 52 g of a residue on drying. A portion of this residue (45 g) was dissolved in 75% methanol and partitioned sequentially with 150 ml (2 aliquots each) of petroleum ether, ethyl acetate and 1-butanol. The residues left on drying of the solvents weighed 1.7 g (petroleum ether), 10.5 g (ethyl acetate), 7.3 g (1-butanol) and 25.4 g (aqueous methanol). In the case of *B. salicifolia*, 500 g of plant material were extracted with 80% methanol, which gave 59.1 g of residue on drying. A portion (40 g) was divided into petroleum ether-soluble (0.4 g), ethyl acetate-soluble (0.9 g), butanol-soluble (1.7 g) and aqueous methanol-soluble extracts (36.8 g). From *H. dulcis*, 182 g of plant material was extracted with 80% methanol, from which 61.2 g of dried residue was obtained. Of this, 50 g was partitioned as indicated above to produce 0.1, 0.2, 2.8 and 46.7 g, respectively, of dried petroleum ether-, ethyl acetate-, butanol- and aqueous methanol-soluble residues. From 104 g of *I. spectabilis* arils, a dried residue weighing 41 g was produced after extraction with 80% methanol, of which 35 g was successively divided into 0.3, 0.9, 1.6 and 32.2 g of dried petroleum ether-, ethyl acetate-, butanol- and aqueous methanol-soluble extracts. The leaves of *S. tinctoria* (361 g) on extraction with 80% methanol afforded a residue weighing 84 g on drying, of which dried petroleum ether- (1.2 g), ethyl acetate- (11.0 g), butanol (18.1 g) and aqueous methanol-soluble (49.6 g) portions were obtained when partitioned as before. From *T. grosvenorii* dried fruits (293 g), a residue of 84 g was obtained after extraction with 80% methanol, from which 50 g was partitioned

into 0.1, 0.4, 5.0 and 44.5 g, respectively, of dried petroleum ether-, ethyl acetate-, butanol- and aqueous methanol-soluble extracts.

Acute toxicity evaluations

Acute toxicity experiments were performed on male Swiss-Webster mice, 4 −6 weeks old. The animals received an orally intubated single dose of each plant extract dispersed in 1% aqueous sodium carboxymethylcellulose, as previously described (Hussain et al., 1986; Compadre et al., 1987). A control group that received only the suspending agent was included in each experiment. Plant extracts were tested at 1 and 2 g/kg body weight. Animals were observed for signs of toxicity and body-weight changes over a period of 14 days after administration, and weight variations were analyzed. None of the extracts tested was found to be acutely toxic for mice at the doses used.

Bacterial mutagenesis assays

Forward mutation assays utilizing *Salmonella typhimurium* strain TM 677, were performed as in a previously described protocol (Pezzuto et al., 1985; Compadre et al., 1987). All plant extracts and their fractions were dissolved in dimethylsulfoxide (20 μl) and evaluated in final concentrations of 0.31, 0.62, 1.25, 2.5 and 5.0 mg/ml. Mutagenicity experiments were performed both in the presence and absence of a 9000 × *g* supernatant fraction (S-9) obtained from livers of Aroclor 1254-pretreated rats. None of the plant extracts tested was found to be mutagenic or bactericidal when evaluated in this manner.

Organoleptic testing

After the safety of plant extracts was established by preliminary safety studies, they were briefly tasted to establish the presence or absence of sweetness by applying approximately 50-mg portions to the tip of the tongue, followed by immediate expectoration. The taster's mouth was rinsed with distilled water before and after each experiment.

Determination of total yields of sugars and polyols

For each of the five sweet plants investigated (*A. hispidum*, *B. salicifolia*, *H. dulcis*, *I. spectabilis* and *T. grosvenorii*), sweetness was found to concentrate exclusively in the final aqueous methanol-soluble extract. These dried extracts, as well as the analogous extract obtained from *S. tinctoria* leaves, were dissolved in water, and the sugars and polyols present in each case were purified and decolorized by passage through short charcoal columns (Norit, F.Q.P., Eastman Organic Chemicals, Rochester, NY), eluted with water and mixtures of water and methanol of decreasing polarity (Hampel and Hawley, 1973). Eluted fractions were monitored by TLC alongside standard sugars, using two chromatographic systems: (a) butanol/acetic acid/water (4:1:2) and (b) butanol/acetic acid/ether/water (9:6:3:1) as developing solvents (Harborne, 1973). TLC plates were visualized after development with a mixture of anisaldehyde/ethanol/sulfuric acid/acetic acid (1:18:1:2), then heated at 100 °C for 10 min (E. Merck

Company, 1980). Sugars were detected as deep violet spots on a light purple background.

In the case of *B. salicifolia* bark, a portion of the final aqueous methanol-soluble extract (11 g) was dissolved in water and passed through a charcoal column (11 g). This column was eluted with water, and a colorless isolate (9.4 g; total yield 10.1% w/w) was collected, which exhibited a single zone by TLC, with an R_f identical to that of sucrose (system a, 0.33; system b, 0.28). The total yields of the combined sugars and polyols of *A. hispidum*, *H. dulcis* and *I. spectabilis* were determined in a similar manner as 6.9, 18.4, and 12.1% w/w, in their plant parts of origin, respectively. For *T. grosvenorii* fruits, 40 g of the final sweet aqueous methanol-soluble extract was adsorbed to silica gel (30 g), then loaded onto a column of silica gel (80 × 7 cm, 250 g), eluted initially by a mixture of chloroform/methanol/water (90:12:1), and thereafter with more polar mixtures of these solvents. All column fractions were examined by TLC, indicating the presence of several sugars, which were decolorized using a charcoal column to furnish a total of 4.1 g of sugars and polyols (total yield of 2.4% w/w). In a similar way, a mixture of monosaccharides was obtained from the final aqueous methanol-soluble extract of *S. tinctoria* leaves in a total yield of 2.0%, also after purification with silica gel and charcoal columns.

Gas chromatography/mass spectrometry

GC/MS experiments were conducted on a Finnigan 4510 Mass Spectrometer/Gas Chromatograph (San José, California), equipped with an INCOS data system. The decolorized final aqueous methanol-soluble extract of each plant was analyzed using both the electron impact (EI) and chemical ionization (CI) modes, using a splitless valve. Prior to injection, thoroughly dried sugar-containing plant extracts (10 mg) and standard sugars (1 mg) were derivatized with Tri-Sil® (0.1 ml, Pierce, Rockford, IL). Mixtures were heated in sealed vials for 15 min at 70 °C.

In the EI mode, samples (1 μl) were injected into a DB-1 fused silica capillary column (J and W Scientific, Folsom, California), 30 m long, 0.25 mm internal diameter and 0.25 μm film thickness. The column temperature was held at 120 °C for 1 min, then programmed at 4 °C/min to 270 °C; the injector, interface line and ion source temperatures were maintained at 220, 280 and 120 °C, respectively. Helium was used as a carrier gas at a head pressure of approximately 0.6 kg/cm^2 and at a flow rate of 2 ml/min. Other parameters were: electron voltage, 70 eV; emission current, 0.25 mA, scan-to-scan time, 1 s; masses scanned 50 – 650 au.

In the CI mode, a DB-5 fused silica capillary column (J and W; 30 m × 0.25 mm i.d.; 0.25 μm film thickness) was used. The initial temperature was kept at 90 °C for 1 min, then raised at a rate of 3 °C/min to 240 °C, and thereafter at 6 °C/min until a final temperature of 280 °C was reached. Ammonia was introduced as the reagent gas at a pressure of 0.4 torr (corrected). Other parameters were similar to those described in the EI mode, except for the following: electron voltage, 90 eV; ion source temperature, 100 °C; scan-to-scan time, 2 s; masses scanned, 110 – 930 au.

TABLE 1

SACCHARIDE AND POLYOL CONSTITUENTS OF SIX PLANT SPECIES, AS DETERMINED BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY[a,b]

Compound	Relative retention time[c]		*Acanthospermum hispidum*	*Boscia salicifolia*	*Hovenia dulcis*	*Inga spectabilis*	*Symplocos tinctoria*	*Thladiantha grosvenorii*
	(a)	(b)						
L-Arabinose	0.66	0.68	–	–	–	0.2	–	0.1
L-Rhamnose	0.69	0.70	–	–	–	–	–	0.1
D-Fructose	0.92	0.90	70.2	–	28.5	21.8	34.6	71.6
D-Galacturonic acid	0.93	0.95	–	–	–	0.2	2.9	–
D-Galactose	0.95	0.98	–	–	2.0	0.1	–	0.2
D-Glucose	1.00	1.00	7.8	–	19.6	7.5	40.4	3.8
D-Mannose	1.03	1.02	1.4	–	0.7	0.8	2.0	0.1
D-Mannitol	1.05	1.03	13.2	–	–	5.3	–	1.4
D-Glucuronic acid methyl ester	1.10	1.06	–	–	25.5	–	–	–
D-Glucuronic acid	1.15	1.09	6.4	–	0.1	–	20.1	3.8
myo-Inositol	1.22	1.20	–	–	–	–	–	8.3
Sucrose	1.77	1.70	1.0	100.0	23.6	64.1	–	1.8
Other[d]	1.50	1.48	–	–	–	–	–	8.8

[a] Calculated as percentage of all sugars and polyols present in a concentration greater than 0.1% w/w. Compounds were identified by direct comparison with authentic samples.

[b] The total w/w yields of sugars and polyols were: *A. hispidum* (leaves), 6.9%; *B. salicifolia* (bark), 10.1%, *H. dulcis* (peduncles), 18.4%; *I. spectabilis* (arils), 12.1%; *S. tinctoria* (leaves), 2.0%; *T. grosvenorii* (fruits), 2.4%.

[c] Analyzed on (a) DB-1 and (b) DB-5 fused silica capillary columns, respectively.

[d] An incompletely characterized disaccharide of D-fructose and D-glucose, that did not correlate to several standard sugars. This tentative identification was achieved using a computerized library (Heller and Milne, 1978, 1980).

For qualitative analysis, retention times were expressed relative to those of glucose. Quantitation of individual sugars and polyols as a percentage of the total saccharide and polyols in a given extract was performed by internal normalization.

Results and discussion

The sugar and polyol contents of the six plants investigated in this study are presented in Table 1. The primary technique for the identification of the individual compounds present in each plant was GC/MS, in which the analytical precision was increased by employing two fused silica capillary columns with different stationary phases, in two different analytical modes (EI and CI). In some instances, identification was further supported by co-TLC of the unknown sugars with authentic compounds. The sugar composition of each species consisted of mixtures of different saccharides with or without polyols (Table 1), with *B. salicifolia* being the only exception, where sucrose was the only sugar detected. The total yield of sucrose in the bark of this plant was calculated as 10.1% w/w, which approaches those of sucrose in sugar cane (15—20% w/w) and sugar beet (10—15% w/w) (Windholz, 1983). Sucrose was also found to occur at a high concentration (7.8% w/w) along with other sugars in the arils of *I. spectabilis*. Therefore, the use of these two species by local populations for their sweet taste, as noted earlier, seems well-founded. The extremely high saccharide levels in *H. dulcis* peduncles (total yield, 18.4%), no doubt account for the selection of a specific epithet for this species denoting sweetness (Hussain et al., 1988). It may also be noted that the dried samples examined of the above three distinctly sweet-tasting species all contained over 10% w/w sugars, whereas the sample of *A. hispidum* dried leaves, which was only moderately sweet, only possessed 6.9% w/w total sugars and polyols. Since the total yield of sugars in the fruits of *T. grosvenorii* (2.4%) was only marginally higher than that of the leaves of *S. tinctoria* leaves, which did not taste appreciably sweet, it may be inferred that the intense sweetness of *T. grosvenorii* fruits is due mainly to the presence of triterpene glycosides such as mogroside V (Takemoto et al., 1983a—c; Makapugay et al., 1985).

The suitability of GC/MS for the analysis of silylated sugars has been demonstrated previously, using glass-packed columns (Sweeley et al., 1966). As a result of the present study, it is clear that GC/MS, involving only minimal work-up, may be used in the future as a simple and convenient means of demonstrating the types of sugars and polyols present in a given sweet plant of interest. It is also of note that, in the El mode, the major mass spectral fragment peaks detected for the standard compounds used in this investigation corresponded with data obtained by other groups (Sweeley et al., 1966; Kochetkov et al., 1968). Difficulties encountered in the detection of molecular peaks of silylated sugars using the EI mode were overcome by the use of CI mass spectrometry. With ammonia as the reagent gas, saccharide and polyol mass spectra were found to exhibit prominent $[M + 18]^+$ and $[M + 1]^+$ fragment peaks, respectively, as previously noted by Loenngren and Svensson (1974).

It may be pointed out that *S. tinctoria* was found to be devoid of any of the sweet dihydrochalcone glycosides previously isolated from three other species of this genus (Tanaka et al., 1980, 1982). Nevertheless, it may be worthwhile to examine other members of this large genus of approximately 350 species (Willis and Airy Shaw, 1980) to see whether this class of sweet dihydrochalcone glucosides have any significance as a chemical marker in the infrageneric classification of *Symplocos*.

Conclusions

An important consideration in the search for sweet-tasting compounds from plants as possible sucrose substitutes is the availability of information on the history of their use for human consumption, either in terms of their edibility or for their alleged medicinal uses. Such records may indicate the potential safety of the plants if used for sweetening purposes. Therefore, it is generally accepted that plants or their products taken orally during the course of human history are likely to be innocuous. All of the six plants selected for the present study had both a history of human consumption and possessed either a reputation for sweetness in the literature or exhibited a sweet taste when collected in the field. Extracts derived from each of the plants studied did not exhibit any acute toxicity for mice or bacterial mutagenicity, thus confirming the probable safety of these species when consumed by local populations in their countries of origin as food, beverage, or medicinal ingredients.

It is well known that many naturally occurring saccharides and polyols other than sucrose are sweet tasting (Kinghorn and Soejarto, 1986) and it may thus be concluded from this study that the sweet tastes of the samples examined of *A. hispidum*, *B. salicifolia*, *H. dulcis* and *I. spectabilis* are due entirely to significant amounts of one or more monosaccharides, disaccharides and polyols, in each case. It can also be asserted that the concentration levels of sugars and polyols in a given plant part should be well over 5% w/w dry weight in order to impart a potent sweet sensation, given that the non-sweet *S. tinctoria* and the moderately sweet *A. hispidum* samples contained, respectively, 2.0% w/w and 6.9% w/w sugars and polyols. Although the sweetness of most fruits is almost certainly due to such high concentrations of sugars, there are some known exceptions to this situation, as exemplified by the fruits of *Thaumatococcus daniellii* (Bennett) Benth. and *Dioscoreophyllum cumminsii* (Stapf) Diels, which are the source of the intensely sweet proteins thaumatin and monellin, respectively (Kinghorn and Soejarto, 1986, 1989). In addition, the sweetness of fruits may also result from the concomitant presence of both sugars and non-caloric intensely sweet compounds, such as in the case of *T. grosvenorii* (Table 1). Therefore, fruits should not be ignored as potential sources of plant-derived non-caloric and non-cariogenic potent sweet substances.

In previous studies, we have demonstrated that the sweetness of certain plants may be attributed to high yields of widely distributed sweet phenylpropanoids, such as *trans*-cinnamaldehyde (Hussain et al., 1986) and *trans*-anethole

(Hussain et al., in press). Thus far, no plant has yet afforded both sweet phenylpropanoid and other intensely sweet constituents, although this is entirely possible in the future. When anethole is present in a plant part, the sweetness sensation may be accompanied by an anise-like smell, thereby facilitating the rapid detection of this sweet compound (Hussain et al., in press). However, there are no modulating sensory characteristics which may indicate whether the sweetness of a plant part is due to the presence of sugars rather than intensely sweet compounds. Therefore, when searching for high potency natural sweeteners, it is recommended that, even if sugars, polyols or phenylpropanoids are detected in a sweet plant part by GC/MS and other methods, appropriate laboratory investigations (extraction, fractionation, toxicity and mutagenicity studies, and compound identification) are conducted to determine conclusively whether or not intensely sweet plant constituents are also present.

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High-Performance Liquid Chromatographic Analysis of the Major Sweet Principle of Lo Han Kuo Fruits

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An analytical high-performance liquid chromatographic (HPLC) procedure has been developed for the triterpene glycoside mogroside V, the principal intensely sweet constituent of Lo Han Kuo fruits [*Thladiantha grosvenori* (Swingle) C. Jeffrey]. Concentration levels of this compound in whole, dried fruits, originating from the People's Republic of China, were in the range 0.81–1.29% w/w. Highest mogroside V levels occurred in the endocarp of the fruits rather than in the peel or in the seeds.

Thladiantha grosvenori (Swingle) C. Jeffrey (formerly *Momordica grosvenori* Swingle) is a vine which is cultivated by the Miao-tze people of Kwangsi Province in the People's Republic of China (Swingle, 1941; Jeffrey, 1979). The fruits of this plant, known to the Chinese as "Lo Han Kuo", are intensely sweet, and also find folklore use in the treatment of colds, sore throats, and stomach and intestinal troubles (Swingle, 1941). Lee (1975) purified a sweet constituent from *T. grosvenori* fruits and estimated its sweetness as being about 150 times that of sucrose. Recently, Takemoto et al. (1983a–c) have elucidated the structures of three sweet triterpene glycoside constituents of *T. grosvenori*, namely, mogroside V, the sweetest and most abundant such compound, and mogrosides IV and VI. Mogroside V has been assigned as the 3-*O*-[β-D-glucopyranosyl (1→6)-β-D-glucopyranoside]-24-*O*-[[β-D-glucopyranosyl (1→2)]-[β-D-glucopyranosyl (1→6)]-β-D-glucopyranoside] of the aglycone mogrol [10α-cucurbit-5-ene-3β,11α,24(*R*),25-tetraol] (Takemoto et al., 1983b,c).

We have studied mogroside V in the course of our research program on intensely sweet plant constituents with potential use as noncariogenic and noncaloric sucrose substitutes and wish to report here a method for the HPLC assay of this compound. Concentration levels of mogroside V in various parts [whole, peel (exocarp and mesocarp), endocarp, seeds] of Lo Han Kuo fruits have been determined. No previous analytical methods for any of the *T. grosvenori* fruit triterpene glycoside sweet constituents appear to have been published, although procedures have been reported for their isolation and purification (Takemoto et al., 1977, 1978; Nippon Chemical Research K.K., 1981a,b) and formulation in an aqueous sweetener mixture (Kamo et al., 1979).

EXPERIMENTAL PROCEDURES

Plant Material. Fruits of *Thladiantha grosvenori* (Swingle) C. Jeffrey (Cucurbitaceae), purchased in both Hong Kong and Chicago, were identified by D.D.S. Both samples were obtained from plants grown in the People's Republic of China.

Isolation of Reference Mogroside V. Gram quantities of mogroside V were generated in the following manner. Powdered *T. grosvenori* fruits (2.75 kg) were percolated with methanol–water (4:1, 20 L), and the percolate was evaporated under reduced pressure at 55 °C to yield a brown gum (800 g). A portion (400 g) was adsorbed to silica gel (Merck, Darmstadt, W. Germany) (1 kg, 0.063–0.2 mm), and loaded into a glass column containing an additional 2.5 kg of silica gel. Separation was carried out by elution with mixtures of chloroform–methanol–water of increasing polarity. Since chloroform has been listed as a carcinogen by the Environmental Protection Agency, chromatographic separations using this solvent were carried out in walk-in fume hood. Elution with chloroform–methanol–water (30:8:1) afforded a series of fractions containing mogroside V, that were combined and dried to produce 22 g of a residue. Aliquots (2 g × 5) of this residue were purified in batch-wise fashion, by dissolution in water (10 mL) and passage through an ion-exchange column (Amberlite IRA-410 C.P., strongly basic, 200 g) (Mallinckrodt Chemical Works, St. Louis, MO) by elution with 250 mL of water. On combining these eluates, partially purified mogroside V (5 g) was thereby obtained free from polar colored contaminants. Final purification of a portion of this material (3.6 g) was effected by isocratic elution over a gravity column containing silica gel (200 g) with chloroform–methanol–water (45:12:2). Altogether, 40 fractions (500 mL each) were collected, and when fractions 28 through 34 were pooled and dried, 2.3 g of pure mogroside V was obtained as a powder: mp 194–196 °C; $[\alpha]^{25}_D$ −16.3° (c 1.6, H_2O) [lit. mp 197–201 °C; $[\alpha]_D$ −9.4° (c 0.5, H_2O) (Takemoto et al., 1983a)]. Purity of the isolate was indicated by its appearance as a single zone after TLC on silica gel GHLF (Analtech, Inc., Newark, DE), using as solvents chloroform–methanol–water (10:10:1) (R_f 0.31), 1-butanol–acetic acid–water (4:1:1) (R_f 0.23), and 1-butanol–ethyl acetate–2-propanol–water (40:20:14:7) (R_f 0.15). Visualization was effected after TLC with 60% w/v sulfuric acid (110 °C, 10 min), whereupon mogroside V appeared as a purple spot in daylight. The molecular weight of this isolate was determined as 1286 dalton by fast-atom bombardment mass spectrometry, a value which is consistent with the elemental composition ($C_{60}H_{102}O_{29}$) of anhydrous mogroside V (Takemoto et al., 1983a). Confirmation of this identification was made by hydrolysis of 200 mg of mogroside V, dissolved in 200 mL of citric acid–disodium hydrogen phosphate buffer at pH 4, with 20 mL of commercial pectinase (Corning Biosystems, Corning, N.Y.) at 37 °C for 140 h. On extraction into chloroform, workup, and crystallization from methanol, 47 mg of mogrol was obtained: mp 120-122 °C; $[\alpha]^{25}_D$ +65.0° (c 0.76, $CHCl_3$). Anal. Found: C, 72.78; H, 10.48. $C_{30}H_{52}O_4 \cdot H_2O$ requires: C, 72.87; H, 10.93%. [Lit. mp 118–119 °C; $[\alpha]^{17}_D$ +70.0° (MeOH), $C_{30}H_{52}O_4$ (Takemoto et al., 1976)]. This hydrolyzate exhibited IR, ^1H NMR, and MS data that were closely comparable to literature data for mogrol (Takemoto et al., 1983b).

HPLC Analysis. HPLC analysis was conducted with a Model 324 gradient liquid chromatograph (Beckman Instruments, Berkeley, CA) equipped with a 421 micro-

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processor controller, Model 100A and 110A pumps, with an added stop-flow valve, and a Model 210 sample injector. A Model LC-85 variable wavelength UV spectrometer with LC-75 Autocontrol (Perkin-Elmer, Norwalk, CT) and an Altex C-R1A recorder (Shimadzu Seisakusho, Kyoto, Japan) was also employed. Solvents used in this study were of HPLC grade and were degassed before use by filtration through an Ultipor NR 0.2 μm filter (Rainin Instrument Co., Woburn, MA) by using a microfiltering apparatus (Sibata, Tokyo, Japan).

The following operating conditions were used for HPLC analysis: column, Zorbax NH_2, 25 cm × 4.6 mm i.d.; particle size, 7 μm (DuPont, Wilmington, DE); eluting solvent, acetonitrile–water (3:1, pH 5 with H_3PO_4); flow-rate, 2 mL/min; wavelength of UV detector, 210 nm; pressure, 1200 psi; sensitivity setting, 0.04 a.u.f.s.; recorder chart speed, 10 mm/min; temperature, ambient.

A Beer's law curve was obtained from triplicate injections of pure mogroside V, dissolved in acetonitrile–water (1:1), at levels of 2.0, 5.0, 10.0, 15.0, and 20.0 μg per injection. Peak height measurement was used and a linear regression equation was established. Mogroside V that eluted from the HPLC column was established as being pure after TLC analysis in the three solvent systems described previously.

Extraction of Plant Material for HPLC. Dried, powdered fruits of *T. grosvenori* (1.0 g) were suspended in water (20 mL), and heated at 45 °C for 4 h. The soluble portion was filtered, the marc washed with water (10 mL), and the filtrates (30 mL) were combined. Further extraction of the marc with water was shown by analytical TLC to provide no additional mogroside V. TLC was carried out according to previously mentioned protocols. When chloroform–methanol–water (10:10:1) was used as solvent, a detection limit of 0.25 μg of mogroside V was possible. The combined aqueous extract was washed with 3 × 30 mL chloroform, and the chloroform layers backwashed with water (30 mL). The total aqueous extraction (60 mL) was then evaporated under reduced pressure at 45 °C to a 10-mL volume. A 2-mL aliquot was then passed into a previously washed precolumn (7 cm × 5 mm, i.d.), packed with C_{18} Phase Bonded Hi-Flosil (Applied Science Laboratories, State College, PA), and polar impurities were removed by elution with 2 mL of water. An extract containing mogroside V was eluted from the precolumn with 3 mL of acetonitrile-water (1:1) and was filtered for HPLC injection.

Triplicate 10-μL injections of each sample extract obtained for *T. grosvenori* whole fruit, peel, endocarp (pulp), and seeds were made, and peak heights were measured. Data were compiled for two *T. grosvenori* samples that were available to this investigation.

Recovery Experiments. Standard mogroside V (7.0 mg) was added to 1.0 g of the exhausted marc from *T. grosvenori* fruits by dissolution in 5 mL of water and evaporation of solvent under reduced pressure at 45 °C. The spiked marc was taken through the entire extraction procedure, the triplicate 10-μL portions of the resulting extract were injected into the HPLC column to determine the percent recovery of mogroside V by the presently described method. The recovery experiment was performed a total of three times.

RESULTS AND DISCUSSION

In Figure 1, an HPLC chromatogram of underivatized mogroside V is shown. A retention time of 6.9 min was obtained for this compound under the chromatographic conditions used. A linear calibration curve ($y = 0.36 + 0.36x$, correlation coefficient $r = 0.99978$) for peak height

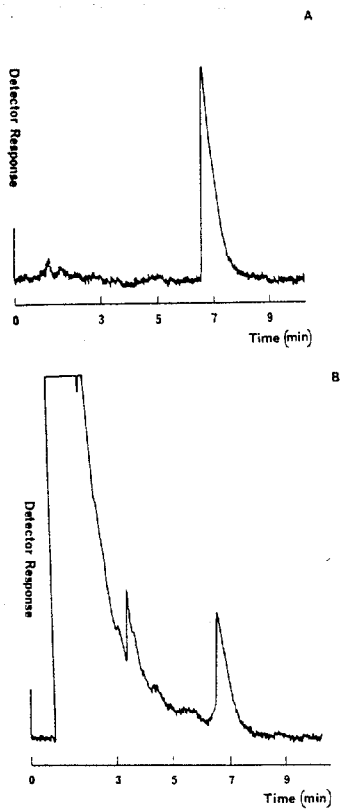

Figure 1. HPLC chromatograms of (A) pure mogroside V and (B) mogroside V as a constituent of an aqueous extract of *Thladiantha grosvenori* whole fruits, purchased in Hong Kong. For operating conditions, see text.

against quantity injected was obtained for mogroside V over the range 2–20 μg. The limit of detection at the 0.04 a.u.f.s. detector setting was 2 μg, as determined by injecting decreasing volumes of a standard 1 mg/mL solution of mogroside V, until the signal-to-noise ratio was about 3:1.

Although we found the isolation of mogroside V from *T. grosvenori* fruits in completely pure form to require considerable attention, it may be seen from Figure 1 that adequate resolution of this compound from other polar constituents was achieved by using the HPLC methodology described here, which involved comparatively little sample cleanup. After controlled experiments to determine the effectiveness of the extraction procedure, the recovery of a standard amount of mogroside V was determined as 82%. The concentration levels of mogroside V in two samples of authenticated *T. grosvenori* fruits available to us were sample 1 (purchased in Hong Kong), whole fruits, 1.29% w/w, peel, 1.26% w/w, endocarp, 1.56% w/w, seeds, 0.24% w/w and sample 2 (purchased in Chicago), whole fruits, 0.80% w/w, peel, 0.81% w/w, endocarp, 1.37% w/w, seeds, 0.51% w/w. Therefore, in both cases, the presence of this compound was observed at the highest concentration levels in the endocarp, when compared with other parts of the fruit. Also, we have been able to confirm the statement of Lee (1975) that the rind (peel) as well as the

pulp of Lo Han Kuo fruits contains a sweet principle.

Takemoto and co-workers (1983a) have reported the isolation of two other sweet triterpene glycoside constituents of *T. grosvenori* fruits in addition to mogroside V, namely, mogrosides IV and VI, which have molecular weights of 1124 and 1448 dalton, respectively. While mogroside VI was found to occur in very small quantities, mogroside IV was obtained in a yield almost as high as mogroside V (Takemoto et al., 1983a). During our work on *T. grosvenori* fruits, we have not observed by analytical TLC or HPLC either mogroside IV or any sweet triterpene glycoside less polar than mogroside V. However, since mogrosides IV and V are clearly separable by reversed-phase HPLC, in which a 25 cm × 4 mm Nucleocil C_{18} column was eluted with 42% ethanol (Takemoto et al., 1983a), it is not expected that significant amounts of mogroside IV in *T. grosvenori* samples will affect the validity of the present HPLC assay for mogroside V.

In other work performed in this laboratory on mogroside V, this compound has been shown to be nonmutagenic and to produce no mortality in acute toxicity experiments on mice at doses up to 2 g/kg body weight and to exhibit an equivalent molar sweetness intensity to the *ent*-kaurene glycoside, stevioside, when tested against a standard sucrose solution by a human taste panel (Kinghorn et al., 1985, unpublished results). These attributes, coupled with the high mogroside V levels in dried *T. grosvenori* fruits that are reported here, could serve to stimulate further study as to the suitability of extracts of the fruit of this plant and its constituents as alternative high-intensity sweeteners. It has been suggested already that *T. grosvenori* may be a suitable species for introduction into the United States (Swingle, 1941).

ACKNOWLEDGMENT

We are grateful to P. But, Chinese University of Hong Kong, for a supply of *T. grosvenori* fruits, and D. L. Smith, University of Utah, for the fast-atom bombardment mass spectrum.

Registry No. Mogroside V, 88901-36-4.

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Changes in the Polypeptide Composition of Peanut (*Arachis hypogaea* L.) Seed during Oil Roasting

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Free amino acids and sugars, released during roasting, are known to be major flavor precursors in roasted peanuts and they give rise to pyrazine compounds via Millard sugar–amine type reaction. In order to identify the protein/polypeptide source of these amino acids, peanut (*Arachis hypogaea L.*) seeds of Virginia, Runner, and Spanish market types were roasted in peanut oil for 0–12 min and protein was extracted and examined by gel filtration and gel electrophoresis. Gel filtration studies indicated that roasting caused a decrease in the methionine-rich proteins and aggregation of arachin proteins. Gel electrophoresis studies also showed a decrease in the methionine-rich protein and their polypeptides. In addition, a polypeptide with a molecular weight of 70 000 also gradually decreased during roasting. In contrast, the protein and polypeptide composition of arachin remained relatively unchanged during the 12-min roasting period. It is suggested that the polypeptide/s of methionine-rich protein may be involved in the formation of pyrazine compounds.

The sugar-amine nonenzymatic browning reaction has been shown to be involved in roasted peanut flavor formation (Pickett and Holley, 1952; Newell et al., 1967; Mason et al., 1969). Free amino acids and free sugars which are released during roasting are known to be major flavor precursors in roasted peanuts (Newell et al., 1967) and they give rise to pyrazine compounds via Millard sugar–amine type reaction (Mason et al., 1967; Johnson et al., 1971; Walradt et al., 1971). Koehler et al. (1969) investigated the pathway for the formation of alkylated

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Stimulation of the Gerbil's Gustatory Receptors by Some Potently Sweet Terpenoids†

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The gerbil was investigated as a model for sweet taste among several highly sweet plant terpenoids and the sweet dihydroisocoumarin phyllodulcin. Although the gerbil's chorda tympani nerve did not respond in electrophysiological experiments to rebaudiosides B and C, steviolbioside, and phyllodulcin, concentration–response curves were obtained for the stimulatory sweeteners hernandulcin, mogroside V, periandrin III, rebaudioside A, and stevioside. These compounds were more effective stimuli in the gerbil than sucrose, in the following order of potency: rebaudioside A = stevioside = periandrin III > hernandulcin > mogroside V > sucrose. In conditioned-taste aversion studies, gerbils trained to avoid these five stimulatory compounds generalized an avoidance to sucrose but not to hydrochloric acid, and except for the perception of a concomitant salty taste, our data show that these substances taste like sucrose to gerbils, as in humans. Support is thus provided for the potential involvement of this methodology to guide the purification of natural sweeteners from plant extracts.

There is an increasing interest in highly sweet nonnutritive and noncariogenic natural sweeteners, and over 50 such substances in more than 15 structural classes are biosynthesized by members of the plant kingdom (Kinghorn and Soejarto, 1986, 1989). Several sweet plant constituents, in either pure or partially purified form, are used commercially as sucrose substitutes in Japan, including the terpenoid glycosides glycyrrhizin, mogroside V, stevioside, and rebaudioside A, the dihydroisocoumarin phyllodulcin, and the protein thaumatin (Ishikawa et al., 1991; Kinghorn and Compadre, 1991). In addition, semisynthetic compounds based on plant constituents such as neohesperidin dihydrochalcone and perillartine are approved sweeteners in a number of countries (Horowitz and Gentili, 1991; Kinghorn and Compadre, 1991).

In electrophysiological gustatory experiments using the Mongolian gerbil, many different classes of naturally occurring and synthetic sweet compounds have been shown to stimulate the animal's taste nerve, including sweet monosaccharides, disaccharides, and polyols, as well as more potently sweet substances such as chlorosucrose, L-cyanosuccinanilic acid, dulcin, sodium saccharin, stevioside, and 6-chloro-D-tryptophan (Jakinovich, 1976, 1981; Jakinovich and Goldstein, 1976; Jakinovich and Oakley, 1976). Furthermore, the behavioral conditioned aversion technique has proven to be successful in rodent studies to identify the taste qualities of many compounds, such as alcohols, amino acids, aspartame, sodium saccharin, and sugars (Garcia et al., 1974; Herness and Pfaffmann, 1986; Jakinovich, 1981, 1982; Kasahara et al., 1987; Kiefer and Lawrence, 1988; Myers et al., 1989; Nachman and Cole, 1971; Ninomiya et al., 1984; Nissenbaum and Scafani, 1987; Nowlis et al., 1980; Pritchard and Scott, 1982; Smith and Theodore, 1984; Spector and Grill, 1988; Steward and Krafczek, 1988; Thomesen et al., 1988). Accordingly, using sweet compounds found to stimulate the gerbil's chorda tympani nerve in electrophysiological experiments, the majority of such substances were found to resemble sucrose in behavioral experiments using the Mongolian gerbil. However, some of these sweet substances were not avoided by animals trained to avoid sucrose, so it cannot be assumed that all compounds that are "sweet" to man are "sweet" to the gerbil (Jakinovich, 1981, 1982a,b).

In an ongoing program to discover novel highly sweet natural products (Kaneda et al., 1992), it has been our practice to subject extracts of sweet-tasting plants to acute toxicity tests in mice and bacterial mutagenicity testing, prior to evaluation for sweetness by human participants. To investigate the possibility of circumventing such a costly and rather inconvenient safety procedure, we have found that a combination of gerbil electrophysiological and conditioned taste aversion experiments could be used in a generally reliable fashion to detect the presence or absence of sweet-tasting terpenoid glycosides in extracts of different polarities of three well-known sweet-tasting plants, namely, *Abrus precatorius*, *Stevia rebaudiana*, and *Thladiantha grosvenorii* (Jakinovich et al., 1990). The present study extends our previous effort by investigating the effect on the gerbil's receptors of several of the purified sweet-tasting diterpene constituents of *S. rebaudiana* (rebaudiosides A–C, stevioside, steviolbioside) (Kinghorn and Soejarto, 1986; Tanaka, 1982) and the major triterpene glycoside sweet principle of *T. grosvenorii* (mogroside V) (Takemoto et al., 1983). In addition, several other pure plant-derived sweeteners have been evaluated in the gerbil model: hernandulcin, a sesquiterpene constituent from *Lippia dulcis* (Compadre et al., 1985); periandrin III, a triterpene glycoside from *Periandra dulcis* (Hashimoto et al., 1982); and phyllodulcin, a dihydroisocoumarin obtained from the crushed or fermented leaves of *Hy-*

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drangea macrophylla var. *Thunbergii* (Arakawa and Nakazaki, 1959; Kinghorn and Soejarto, 1986). Since many of these potent natural sweeteners were observed in the present study to stimulate the gerbil's chorda tympani nerve and to taste like sucrose in behavioral experiments, further support has been obtained for the inclusion of experiments on the gerbil in the fractionation of sweet-tasting plants, thereby partially offsetting the need for human volunteer subjects.

MATERIALS AND METHODS

General Procedures. Melting points were determined on a Kofler hot-stage instrument and are uncorrected. Optical rotations, UV, IR, ^{1}H NMR, ^{13}C NMR, and low-resolution mass spectrometry were performed as described previously (Kaneda et al., 1992). Analytical TLC was carried out on Merck silica gel G plates, with 250 μm thick layers, which were visualized in short-wave UV light and/or after spraying with 60% w/v H_2SO_4 and heating at 110 °C for 10 min. Where suitable, HPLC analysis was performed to further verify purity (Makapugay et al., 1984, 1985; Compadre et al., 1987).

Test Compounds. Hernandulcin (1) was synthesized in the racemic form by directed-aldol condensation from 3-methyl-2-cyclohexen-1-one and 6 methyl-5-hepten-2-one and purified as previously described. This compound was indistinguishable spectroscopically and chromatographically from its naturally occurring (+) form, (6*S*,1′*S*)-hernandulcin (Compadre et al., 1985, 1987). Mogroside V (2) was purified from Lo Han Kuo fruits [*T. grosvenorii* (Swingle) C. Jeffrey; recently renamed *Siraitia grosvenorii* (Swingle) C. Jeffrey] (Kinghorn and Compadre, 1991) and characterized as described previously (Makapugay et al., 1985). The trisodium salt of periandrin III (3) was generously donated by Yamasa Shoyu Co., Ltd. (Chosi, Chiba, Japan) and, after conversion to the parent compound and workup, exhibited physical and spectroscopic data identical to published values for periandrin III (3) (Hashimoto et al., 1982).

The dihydroisocoumarin, phyllodulcin (4), was isolated from the crushed leaves of *H. macrophylla* Seringe var. *Thunbergii* (Siebold) Makino, kindly supplied by the late Prof. T. Takemoto. An initial methanol–water (4:1) extract was prepared from the dried milled plant material (7 kg) from which the solvent was removed. On suspension in water and partitioning with ethyl acetate, the ethyl acetate residue (350 g) was purified by gravity column chromatography over silica gel (Merck, Darmstadt, Germany), and phyllodulcin was eluted with mixtures of petroleum ether–chloroform in proportions of 13:7 and 1:1. Isolated phyllodulcin (4, 90 g, 1.28% w/w) was recrystallized as white needles from chloroform–petroleum ether [mp 118–119 °C, $[\alpha]^{25}_D$ +80.5° (*c* 2.9, $CHCl_3$) [lit. mp 119–121 °C; $[\alpha]^{25}_D$ +70.7–80.8° (*c* 1.02, Me_2CO)] (Arakawa and Nakazaki, 1959)] and exhibited spectroscopic data closely comparable to published values for this compound (Suzuki et al., 1978). The identity of 4 as phyllodulcin was confirmed by direct comparison (mmp, EI-MS, ^{1}H NMR, co-TLC) to a reference sample kindly supplied by Prof. M. Yamamoto.

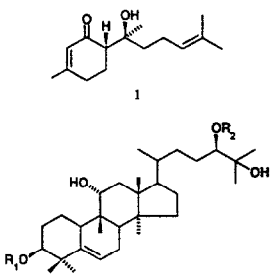
Rebaudioside A (5), rebaudioside C (7), and stevioside (9) were isolated and characterized from *S. rebaudiana* (Bertoni) Bertoni leaves, as described previously. Rebaudioside B (6) and steviolbioside (8) were obtained from compounds 5 and 9, respectively, by alkaline hydrolysis. All five of these sweet diterpene glycosides exhibited physical and spectroscopic data consistent with literature values (Makapugay et al., 1984).

Prior to being used in the present study, all compounds were tested for purity by analytical TLC and/or HPLC. The structures of test compounds 1–9 are shown in Figure 1.

Experimental Animals. Mongolian gerbils (*Meriones unguiculatus*) were obtained from Tumblebrook Farms, West Brookfield, MA. For electrophysiological experiments, male and female animals, and were less than 1 year old and weighing 50–70 g, were used. For behavioral experiments, male gerbils aged 7–12 weeks were utilized and were 50–60 g in weight.

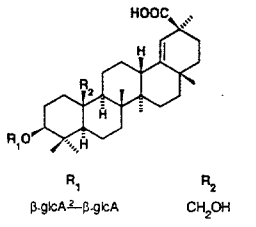
Methods. *Electrophysiological Methods.* (*a*) *Anesthetic.* Gerbils were injected with ketamine as the primary anesthetic because it produces complete anesthesia in 5–10 min. The ketamine (100 mg/mL) was injected at a dose of 330 mg/kg into the gerbil's thigh muscle. If an animal required further anesthetic during the experiment, sodium pentobarbital (5 mg/mL; 0.15 mL) injected intraperitoneally was employed (Somenerain and Jakinovich, 1990).

(*b*) *Electrophysiology.* Each animal was secured to a head-holder (Oakley and Schaffer, 1978) which immobilized the skull.

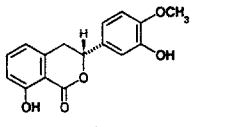


1

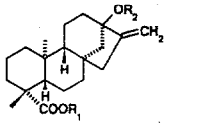
2. R_1: β-glc^{2-}β-glc; R_2: β-glc^{2-}β-glc (β-glc^{3}) + β-glc



3. R_1: β-glcA^{2-}β-glcA; R_2: CH_2OH



4



	R_1	R_2
5	β-glc	β-glc^{2-}β-glc (β-glc^{3}) + β-glc
6	H	β-glc^{2-}β-glc (β-glc^{3}) + β-glc
7	β-glc	β-glc^{2-}α-rha (β-glc^{3}) + β-glc
8	H	β-glc^{2-}β-glc
9	β-glc	β-glc^{2-}β-glc

Figure 1. Chemical structures of the highly sweet terpenoids (1–3, 5–9) and the dihydroisocoumarin (4) investigated in this study. Sugar units: β–glc = β-D-glucopyranosyl; β-glcA = β-D-glucuronopyranosyl; α-rha = α-L-rhamnopyranosyl.

The method for exposing and recording from the intact chorda tympani nerve has been reported in detail (Somenerain and Jakinovich, 1990).

(c) *Stimulation.* Chemical stimulation of the tongue was effected by a gravity-flow funnel-tubing system through which deionized water flowed continuously (0.13–0.17 mL/s). Test solutions (2–4 mL) were alternated with water without interruption of the flow. The temperatures of the water and the taste solutions were identical, 25 ± 1 °C. Each compound was tested twice, before and after a standard. Whenever the standard solution elicited responses differing by more than 15%, all interjacent responses were rejected.

(d) *Taste Solutions.* All compounds were dissolved in deionized tap water (>1 megohm). When not used immediately, the sweetener solutions were stored in frozen form or at 2 °C for later use, when they were brought to room temperature.

(e) *Mixtures.* To determine if nonstimulating sweeteners were interacting with the sweetener taste receptor sites, responses to mixtures of sucrose and a nonstimulating sweetener and of rebaudioside A and a nonstimulating sweetener were compared to responses to sucrose and rebaudioside A (Jakinovich, 1981, 1983; Vlahopoulos and Jakinovich, 1986).

Behavioral Methods. (a) *General Scope of Study.* This behavior study comprised two sets of experiments, with the first to determine how the gerbils perceived the taste of mogroside V (2, 0.001 M) and stevioside (9, 0.002 M). The second set of experiments dealt with the perception of the taste by the gerbil of hernandulcin (1, 0.01 M), periandrin III (3, sodium salt) (0.003 M), and rebaudioside A (5, 0.002 M). The concentrations used were the CR_{50}'s (concentrations that produced half-maximal responses), as determined from the appropriate electrophoretic concentration–response curve, or else the maximum solubility obtained in water.

(b) *Conditioned Taste Aversion.* The following training procedures were used in all experiments:

(1) *Water Intake Training.* One day after arrival, the animals were housed in individual plastic cages with wood chip bedding, rather than in individual wire-bottom cages, to avoid health problems (Jakinovich, 1981, 1982; Jakinovich et al., 1990; Myers et al., 1989). Two days later, all test animals were placed on a drinking schedule whereby they received deionized water twice daily (from 09:00 to 10:00 a.m. and from 3:00 to 4:00 p.m.). Animals were fed Purina Rat Chow (Ralston Purina Co.) *ad libitum.*

(2) *Conditioned Avoidance Training.* After 6 days of the water training, the animals were randomly divided into three groups of 12 each, with the first group trained to avoid mogroside V (2, 0.001 M), the second group trained to avoid stevioside (9, 0.002 M), and a control group trained to avoid water. The mogroside V (0.001 M) and stevioside (0.002 M) solutions were used as conditioning solutions in the following manner: On the Friday of each of the *consecutive* weeks, during the usual morning drinking period one group of gerbils was offered a drinking bottle containing mogroside V, one group was offered stevioside, and the third group, acting as control, received only water. These solutions were offered for 15 min during the morning watering period. When each animal finished drinking, its drinking tube was again placed in its mouth, leaving a few drops behind, and, immediately after that, the gerbil was injected with LiCl (0.3 M) at 1% of its body weight. Shortly after the injections, the animals showed lethargic appearances which lasted for several hours. This entire avoidance training procedure, as described above, was repeated on the following Friday, and on the Friday of each consecutive week.

(3) *Conditioned Avoidance Testing.* On the Monday of the third week, having allowed 2 days for the animals to recuperate, half of the animals in each group were offered water bottles containing sucrose (0.03 M), while the other half received bottles containing NaCl solution (0.01 M). Then, the following morning the animals received these two solutions in reverse order. On Wednesday and Thursday, the above procedure was repeated. Measurements of the amounts of particular fluids consumed were made by weighing the drinking bottles immediately after each animal stopped drinking. During the afternoon drinking period, all animals received only deionized water.

On the Monday of the fourth week, 50% of the animals in each group were offered water bottles containing HCl (0.01 M), while the other half received bottles of quinine hydrochloride solution (0.001 M). Next, on the following morning, the animals received those solutions in reverse order. On Wednesday and Thursday, the above procedure was repeated.

To strengthen and reinforce the aversion, the entire conditioning and testing was repeated. The entire above-indicated behavioral procedure was repeated with solutions of hernandulcin (1, 0.01 M), periandrin III (3, sodium salt, 0.003 M), and rebaudioside A (5, 0.002 M).

(c) *Statistics.* One-way analyses of variance (ANOVA) were applied to the behavioral results. When significant differences were observed, pairwise analyses (*t*-test) were performed between the control and experimental groups (see Results).

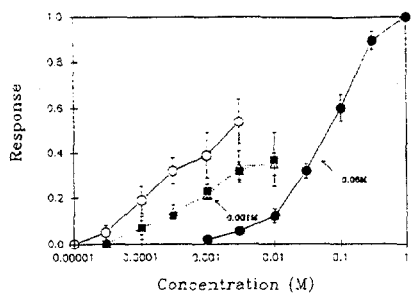


Figure 2. Integrated neural discharge from the gerbil's chorda tympani nerve in response to various concentrations (log scale) of purified rebaudioside A (△), Nutrilite stevioside (impure commercial source) (○), purified stevioside (■), and sucrose (●). The CR_{50}'s are shown. Bars indicate ±2SE; N for sucrose = 14, N for purified stevioside = 5, and N for rebaudioside A = 8.

RESULTS

No Electrophysiological Responses. The following compounds did not produce responses in the gerbil's chorda tympani nerve at the concentrations used: phyllodulcin (4), rebaudioside B (6), rebaudioside C (7), and steviolbioside (8). Their maximum solubility in water ranged from 1×10^{-3} to 5×10^{-4} M.

Electrophysiological Responses—Concentration–Response Curves Determined. Hernandulcin (1), mogroside V (2), periandrin III (3, sodium salt), rebaudioside A (5), and stevioside (9) produced responses in the gerbil's chorda tympani nerve. The maximum solubility of the compounds in water was around 0.01 M, which was lower than that of sucrose in all cases. Responses to two compounds, rebaudioside A and stevioside, that produced concentration–response curves with a shape similar to that of sucrose, are shown in Figure 2. These concentration curves exhibited a sigmoidal shape with a maximum response (R_{max}) evident, which is a characteristic of a normal neural sweetener taste response (Jakinovich and Sugarman, 1989). The R_{max} values of rebaudioside A and stevioside were 0.4 (sucrose R_{max}, 1.0). The previously published concentration–response curve of stevioside obtained from a commercial source (Nutrilite Products Inc., Buena Park, CA) is included in Figure 2 for comparison purposes (Jakinovich, 1981). Since R_{max} values were determined, CR_{50} data could be used as one measure of potency (Jakinovich, 1976), and the results obtained were rebaudioside A CR_{50} = 0.001 M, stevioside CR_{50} = 0.001 M, and sucrose CR_{50} = 0.06 M. Another measure of potency, the K_d value (dissociation constant, representing the efficacy of the sweet compounds) (Biedler, 1954), was determined for three sweeteners using the reciprocal plot, as expressed in Figure 3 (sucrose, 0.07 M; stevioside, 0.0013 M; rebaudioside A, 0.0014 M). A third measure of potency, threshold value, was determined directly from Figure 2

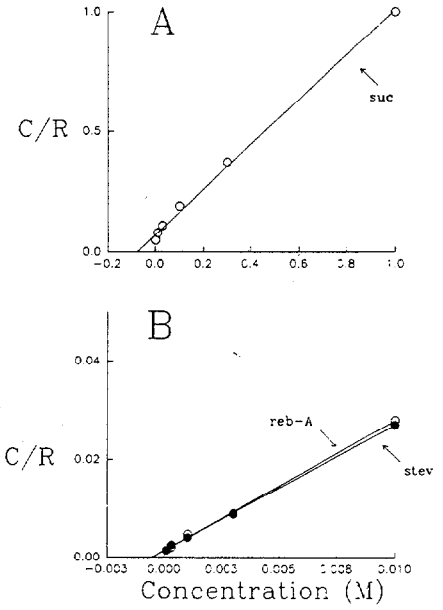


Figure 3. (A) Reciprocal plot of gerbil integrated chorda tympani nerve responses to sucrose (suc, O). (B) Reciprocal plot of gerbil integrated chorda tympani nerve responses to rebaudioside A (reb-A, O) and stevioside (stev, ●). C = concentration, R = response, slope = $1/R_{max}$, K_d/R_{max} = y intercept, and $-K_d$ = x intercept.

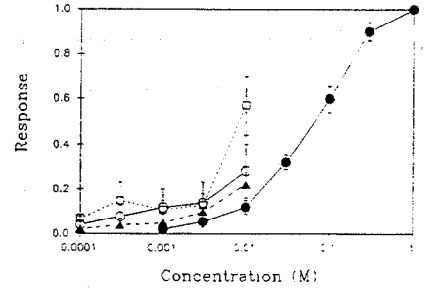


Figure 4. Integrated neural discharge from the gerbil's chorda tympani nerve in response to various concentrations (log scale) of hernandulcin (▲), mogroside V (□), periandrin III (sodium salt) (O), and sucrose (●). Bars indicate ±2SE; N for sucrose = 14, N for hernandulcin = 5, N for mogroside V = 6, and N for periandrin III (sodium salt) = 5.

(sucrose, 0.003 M; stevioside, 0.0001 M; rebaudioside A, 0.0001 M).

We were unable to obtain complete sigmoidally shaped (with R_{max}) concentration–response curves for three compounds, hernandulcin (1), mogroside V (2), and periandrin III (3; sodium salt) (Figure 4). However, since the R_{max}'s were not present, the potency of each compound was determined from its K_d in the reciprocal plot (Figure 5) (Beidler, 1954), with the following values obtained: periandrin III, sodium salt, K_d = 0.0006 M; mogroside V, K_d = 0.003 M; hernandulcin, K_d = 0.002 M. Thresholds were determined from Figure 4: periandrin III (sodium salt) threshold, 0.0001 M; mogroside V threshold, 0.0001 M; and hernandulcin threshold, 0.0003 M.

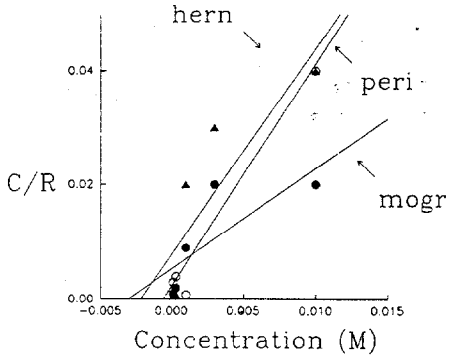


Figure 5. Reciprocal plot of gerbil integrated chorda tympani nerve responses to hernandulcin (hern, ▲), mogroside V (mogro, ●), and periandrin III (sodium salt) (peri, O).

Mixtures. The taste responses to sucrose or rebaudioside A were not influenced by the presence of a nonstimulating sweetener.

Behavioral Responses. In all cases, gerbils trained to avoid an intense natural sweetener consumed significantly less sucrose (0.03 M) than water (Table I). Concerning saltiness, gerbils trained to avoid hernandulcin, rebaudioside A, and periandrin III (sodium salt) consumed significantly less NaCl (0.01 M) than water. For sourness, intakes of HCl (0.01 M) by all trained gerbils were no different from water. Finally, for bitterness, gerbils trained to avoid mogroside V, hernandulcin, rebaudioside A, and periandrin III (sodium salt) consumed significantly less quinine hydrochloride (0.001 M) than water.

DISCUSSION

In the present gerbil electrophysiological investigation, the CR_{50} in the standard sucrose concentration–response curve was 0.06 M, which is in general agreement with our past gerbil studies in which the sucrose CR_{50} values ranged from 0.015 to 0.05 M (Somenerain and Jakinovich, 1990). Another consistency was that the sucrose concentration–response curve was sigmoidally shaped and reached an R_{max} (Figure 2). However, it was found in this study that the plant-derived sweeteners phyllodulcin (4), rebaudiosides B and C (6, 7), and steviolbioside (8) did not stimulate the gerbil's chorda tympani nerve in electrophysiological experiments (Table II). These observations are consistent with our previous gerbil study in which it was discerned that a lack of response in this regard was evident with the following natural and artificial potently sweet compounds: aspartame, beryllium acetate, furan acrylonitrile, 4-(methoxymethyl)-1,4-cyclohexadiene-1-carboxaldehyde *syn*-oxime, monellin, 5-nitro-2-propoxyaniline, and perillartine (Jakinovich, 1981).

The electrophysiological concentration–response curves of the purified stevioside (9) and rebaudioside A (5) used in this investigation were also sigmoidally shaped and reached an R_{max} of 0.4 (Figure 2). The CR_{50} of both these compounds was 0.001 M. The concentration–response curve of our previously published work using a commercially available stevioside sample is anomalous because it did not reach an R_{max} and was not sigmoidal (Jakinovich, 1981). This distortion is probably due to the presence of unspecified impurities, which are also thought to be responsible for distorted concentration–response curves in the gerbil's chorda tympani nerve responses reported for methyl β-D-galactopyranoside (Jakinovich, 1985) or in human psychophysics studies for D-ribitol (Jakinovich and

Table I. Amounts (Milliliters ± Standard Error) of Test and Control Solutions Consumed by Gerbils Trained To Avoid Highly Sweet Plant-Derived Constituents

		test solution			
conditioning solution	control solution	0.03 M sucrose	0.1 M NaCl	0.01 M HCl	0.001 M quinine hydrochloride
mogroside V (0.001 M)		0.66 ± 0.21[a]	3.19 ± 0.27	1.22 ± 0.22	0.54 ± 0.13[a]
stevioside (0.02 M)		0.39 ± 0.06[a]	3.32 ± 0.31	1.06 ± 0.17	1.01 ± 0.21
	water–sucrose	2.07 ± 0.29			
	water–NaCl		2.71 ± 0.18		
	water–HCl			1.63 ± 0.07	
	water–quinine hydrochloride				1.23 ± 0.12
hernandulcin (0.01 M)		0.95 ± 0.22[a]	1.52 ± 0.35[a]	1.6 ± 0.38	0.76 ± 0.17[a]
rebaudioside A (0.02 M)		0.21 ± 0.01[a]	1.05 ± 0.31[a]	1.1 ± 0.16	0.89 ± 0.23[a]
periandrin III (sodium salt) (0.003 M)		1.14 ± 0.27[a]	1.12 ± 0.34[a]	1.6 ± 0.31	0.90 ± 0.19[a]
	water–sucrose	1.81 ± 0.16			
	water–NaCl		2.38 ± 0.38		
	water–HCl			1.85 ± 0.25	
	water–quinine hydrochloride				1.58 ± 0.18

[a] Significant difference, $p > 0.01$; $N \approx 12$.

Table II. Gerbil Electrophysiological Responses to Stimulatory Plant-Derived Sweeteners[a,b]

compound	threshold, M	K_d, M	CR_{50}, M	sweetness to humans[c] (× sucrose)	ref
sucrose	0.003	0.07	0.06	1	Beck (1974)
mogroside V	0.0001	0.002	ND[d]	340	Takemoto et al. (1983)
hernandulcin	0.0003	0.003	ND[d]	1,500	Compadre et al. (1985)
periandrin III (sodium salt)	0.0001	0.0006	ND[d]	80	Kinghorn and Soejarto (1986)
rebaudioside A	0.0001	0.0006	0.001	240	Kasai et al. (1981)
stevioside	0.0001	0.0006	0.001	140	Kasai et al. (1981)

[a] Test compounds are ranked according to K_d. [b] The following compounds in the study were nonstimulatory at the doses specified: phyllodulcin (5×10^{-4} M); rebaudioside B (1×10^{-3} M); rebaudioside C (1×10^{-3} M); steviolbioside (1×10^{-3} M). [c] Figures are expressed as approximate sweetness intensities on a weight comparison to sucrose. [d] ND, not determined.

Sugarman, 1989). The reduced R_{max}'s observed in the stevioside and rebaudioside A concentration–response curves when compared to that of sucrose suggested that stevioside and rebaudioside A either are partial agonists at the receptor site or else bind at a different receptor site from sucrose (Ariëns et al., 1964). Complete concentration–response curves were not obtained for mogroside V (2), periandrin III (3; used as the sodium salt), and hernandulcin (1). In spite of this, it was possible to rank the potency of these sweeteners as gustatory stimuli in the gerbil on the basis of their determined K_d values in the following order of decreasing magnitude: rebaudioside A = stevioside = periandrin III (sodium salt) > hernandulcin > mogroside V > sucrose (Table II). A similar ranking list of potency was obtained by considering threshold and CR_{50} values.

The results of the behavioral experiments showed that the gerbil's taste responses to hernandulcin, mogroside V, periandrin III (sodium salt), rebaudioside A, and stevioside resemble their effects in humans. To the human, representatives of these highly sweet natural sweeteners, for which hedonic data have been reported, have been found to taste either sweet (resembling the taste of sucrose) or sweet-bitter (resembling the taste of sucrose and quinine) (Compadre et al., 1985; Schiffman et al., 1979). In the human, all five of these natural sweeteners are sweeter than sucrose, in the following order of potency of sweetness intensity: hernandulcin > mogroside A > rebaudioside A > stevioside > periandrin III > sucrose (Table II) (Compadre et al., 1985; Kasai et al., 1981; Kinghorn and Soejarto, 1986; Takemoto et al., 1983). When the two *ent*-kaurene glycoside sweeteners in this group of stimulatory compounds are considered structurally, rebaudioside A possesses one more glucopyranosyl moiety in its C-13-affixed saccharide unit than stevioside, which accordingly confers greater sweetness and more pleasant hedonic attributes for humans. However, the sweetness potency of rebaudioside A is greatly diminished in human subjects either by removal of the glucose attached to C-19 or by substitution of a glucose moiety by rhamnose in the C-13 sugar unit, as in rebaudiosides B and C, respectively (Kinghorn and Soejarto, 1986; Tanaka, 1982).

In conclusion, a combination of two well-established gerbil electrophysiological and behavioral assays has been applied to a structurally diverse group of terpenoids that are highly sweet to humans, constituted by the bisabolane sesquiterpene hernandulcin, the cucurbitane triterpene glycoside mogroside V, the oleanane-type triterpene glycoside periandrin III, and the *ent*-kaurene-type diterpene glycosides rebaudioside A and stevioside. It is significant that the most abundant sweet constituents of *T. grosvenorii* fruits (mogroside V) and *S. rebaudiana* leaves (stevioside and rebaudioside A) have been found to stimulate the gerbil's taste receptors in this study, since we have earlier shown that extracts containing these compounds also gave positive data (Jakinovich et al., 1990). Although these gerbil assays do not respond to all classes of compounds perceived as sweet by humans, they do seem to have validity for evaluating sweet-tasting terpenoids of plant origin.

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High-Performance Liquid Chromatographic Analysis of the Major Sweet Principle of Lo Han Kuo Fruits

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An analytical high-performance liquid chromatographic (HPLC) procedure has been developed for the triterpene glycoside mogroside V, the principal intensely sweet constituent of Lo Han Kuo fruits [*Thladiantha grosvenori* (Swingle) C. Jeffrey]. Concentration levels of this compound in whole, dried fruits, originating from the People's Republic of China, were in the range 0.81–1.29% w/w. Highest mogroside V levels occurred in the endocarp of the fruits rather than in the peel or in the seeds.

Thladiantha grosvenori (Swingle) C. Jeffrey (formerly *Momordica grosvenori* Swingle) is a vine which is cultivated by the Miao-tze people of Kwangsi Province in the People's Republic of China (Swingle, 1941; Jeffrey, 1979). The fruits of this plant, known to the Chinese as "Lo Han Kuo", are intensely sweet, and also find folklore use in the treatment of colds, sore throats, and stomach and intestinal troubles (Swingle, 1941). Lee (1975) purified a sweet constituent from *T. grosvenori* fruits and estimated its sweetness as being about 150 times that of sucrose. Recently, Takemoto et al. (1983a–c) have elucidated the structures of three sweet triterpene glycoside constituents of *T. grosvenori*, namely, mogroside V, the sweetest and most abundant such compound, and mogrosides IV and VI. Mogroside V has been assigned as the 3-*O*-[β-D-glucopyranosyl (1→6)-β-D-glucopyranoside]-24-*O*-[[β-D-glucopyranosyl (1→2)]-[β-D-glucopyranosyl (1→6)]-β-D-glucopyranoside] of the aglycone mogrol [10α-cucurbit-5-ene-3β,11α,24(*R*),25-tetraol] (Takemoto et al., 1983b,c).

We have studied mogroside V in the course of our research program on intensely sweet plant constituents with potential use as noncariogenic and noncaloric sucrose substitutes and wish to report here a method for the HPLC assay of this compound. Concentration levels of mogroside V in various parts [whole, peel (exocarp and mesocarp), endocarp, seeds] of Lo Han Kuo fruits have been determined. No previous analytical methods for any of the *T. grosvenori* fruit triterpene glycoside sweet constituents appear to have been published, although procedures have been reported for their isolation and purification (Takemoto et al., 1977, 1978; Nippon Chemical Research K.K., 1981a,b) and formulation in an aqueous sweetener mixture (Kamo et al., 1979).

EXPERIMENTAL PROCEDURES

Plant Material. Fruits of *Thladiantha grosvenori* (Swingle) C. Jeffrey (Cucurbitaceae), purchased in both Hong Kong and Chicago, were identified by D.D.S. Both samples were obtained from plants grown in the People's Republic of China.

Isolation of Reference Mogroside V. Gram quantities of mogroside V were generated in the following manner. Powdered *T. grosvenori* fruits (2.75 kg) were percolated with methanol–water (4:1, 20 L), and the percolate was evaporated under reduced pressure at 55 °C to yield a brown gum (800 g). A portion (400 g) was adsorbed to silica gel (Merck, Darmstadt, W. Germany) (1 kg, 0.063–0.2 mm), and loaded into a glass column containing an additional 2.5 kg of silica gel. Separation was carried out by elution with mixtures of chloroform–methanol–water of increasing polarity. Since chloroform has been listed as a carcinogen by the Environmental Protection Agency, chromatographic separations using this solvent were carried out in walk-in fume hood. Elution with chloroform–methanol–water (30:8:1) afforded a series of fractions containing mogroside V, that were combined and dried to produce 22 g of a residue. Aliquots (2 g × 5) of this residue were purified in batch-wise fashion, by dissolution in water (10 mL) and passage through an ion-exchange column (Amberlite IRA-410 C.P., strongly basic, 200 g) (Mallinckrodt Chemical Works, St. Louis, MO) by elution with 250 mL of water. On combining these eluates, partially purified mogroside V (5 g) was thereby obtained free from polar colored contaminants. Final purification of a portion of this material (3.6 g) was effected by isocratic elution over a gravity column containing silica gel (200 g) with chloroform–methanol–water (45:12:2). Altogether, 40 fractions (500 mL each) were collected, and when fractions 28 through 34 were pooled and dried, 2.3 g of pure mogroside V was obtained as a powder: mp 194–196 °C; $[\alpha]^{25}_D$ −16.3° (c 1.6, H_2O) [lit. mp 197–201 °C; $[\alpha]_D$ −9.4° (c 0.5, H_2O) (Takemoto et al., 1983a)]. Purity of the isolate was indicated by its appearance as a single zone after TLC on silica gel GHLF (Analtech, Inc., Newark, DE), using as solvents chloroform–methanol–water (10:10:1) (R_f 0.31), 1-butanol–acetic acid–water (4:1:1) (R_f 0.23), and 1-butanol–ethyl acetate–2-propanol–water (40:20:14:7) (R_f 0.15). Visualization was effected after TLC with 60% w/v sulfuric acid (110 °C, 10 min), whereupon mogroside V appeared as a purple spot in daylight. The molecular weight of this isolate was determined as 1286 dalton by fast-atom bombardment mass spectrometry, a value which is consistent with the elemental composition ($C_{60}H_{102}O_{29}$) of anhydrous mogroside V (Takemoto et al., 1983a). Confirmation of this identification was made by hydrolysis of 200 mg of mogroside V, dissolved in 200 mL of citric acid–disodium hydrogen phosphate buffer at pH 4, with 20 mL of commercial pectinase (Corning Biosystems, Corning, N.Y.) at 37 °C for 140 h. On extraction into chloroform, workup, and crystallization from methanol, 47 mg of mogrol was obtained: mp 120-122 °C; $[\alpha]^{25}_D$ +65.0° (c 0.76, $CHCl_3$). Anal. Found: C, 72.78; H. 10.48. $C_{30}H_{52}O_4 \cdot H_2O$ requires: C, 72.87; H, 10.93%. [Lit. mp 118–119 °C; $[\alpha]^{17}_D$ +70.0° (MeOH), $C_{30}H_{52}O_4$ (Takemoto et al., 1976)]. This hydrolyzate exhibited IR, ^1H NMR, and MS data that were closely comparable to literature data for mogrol (Takemoto et al., 1983b).

HPLC Analysis. HPLC analysis was conducted with a Model 324 gradient liquid chromatograph (Beckman Instruments, Berkeley, CA) equipped with a 421 micro-

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processor controller, Model 100A and 110A pumps, with an added stop-flow valve, and a Model 210 sample injector. A Model LC-85 variable wavelength W spectrometer with LC-75 Autocontrol (Perk&Elmer, Norwalk, CT) and an Altex C-R1A recorder (Shimadzu Seisakusho, Kyoto, Japan) was also employed. Solvents used in this study were of HPLC grade and were degassed before use by filtration through an Ultipor NR 0.2 pm filter (Rainin Instrument Co., Wobum, MA) by using a microfiltering apparatus (Sibata, Tokyo, Japan).

The following operating conditions were used for HPLC analysis: column, Zorbax NH_2, 25 cm X 4.6 mm i.d.; particle size, 7 μm (DuPont, Wilmington, DE); eluting solvent, acetonitrile-water (3:1, pH 5 with H_3PO_4); flow-rate, 2 mL/min; wavelength of UV detector, 210 nm; pressure, 1200 psi; sensitivity setting, 0.04 a.u.f.s.; recorder chart speed, 10 mm/min; temperature, ambient.

A Beer's law curve was obtained from triplicate injections of pure mogroside V, dissolved in acetonitrile-water (1:1), at levels of 2.0, 5.0, 10.0, 15.0, and 20.0 μg per injection. Peak height measurement was wed and a linear regression equation was established. Mogroside V that eluted from the HPLC column was established as being pure after TLC analysis in the three solvent systems described previously.

Extraction of Plant Material for HPLC. Dried, powdered fruits of *T.* grosuenori (1.0 g) were suspended in water (20 mL), and heated at 45 °C for 4 h. The soluble portion was filtered, the marc washed with water (10 mL), and the filtrates (30 mL) were combined. Further extraction of the marc with water was shown by analytical TLC to provide no additional mogroside V. TLC was carried out according to previously mentioned protocols. When chloroform-methanol-water (10:10:1) was used as solvent, a detection limit of 0.25 μg of mogroside V was possible. The combined aqueous extract was washed with 3 X 30 mL chloroform, and the chloroform layers back-washed with water (30 mL). The total aqueous extraction (60 mL) was then evaporated under reduced pressure at 45 °C to a 10-mL volume. A 2-mL aliquot was then passed into a previously washed precolumn (7 cm x 5 mm, i.d.), packed with C_{18} Phase Bonded Hi-Flosil (Applied Science Laboratories, State College, PA), and polar impurities were removed by elution with 2 mL of water. An extract containing mogroside V was eluted from the precolumn with 3 mL of acetonitrile-water (1:1) and was filtered for HPLC injection.

Triplicate 10-μL injections of each sample extract obtained for *T. grosuenori* whole fruit, peal, endocarp (pulp), and seeds were made, and peak heights were measured. Dam were compiled for two *T.* grosuenori samples that were available to this investigation.

Recovery Experiments. Standard mogroside V (7.0 mg) was added to 1.0 g of the exhausted marc from *T. grosvenori* fruits by d&solution in 5 mL of water and evaporation of solvent under reduced pressure at 45 "C. The spiked marc was taken through the entire extraction procedure, the triplicate 10-μL portions of the resulting extract were injected into the HPLC column to determine the percent recovery of mogroside V by the presently described method. The recovery experiment was performed a l of three times.

RESULTS AND DISCUSSION

In Figure 1, an HPLC chromatogram of underivatized mogroside V is shown. A retention time of 6.9 min was obtained for this compound under the chromatographic conditions used. A linear calibration curve ($y = 0.36 + 0.36x$, correlation coefficient $r = 0.99978$) for peak height against quantity injected was obtained for mogroside V over the range 2–20 pg. The limit of detection at the 0.04 a.u.f.s. detector setting was 2 μg, as determined by injecting decreasing volumes of a standard 1 mg/mL solution of mogroside V, until the signal-to-noise ratio was about 3:1.

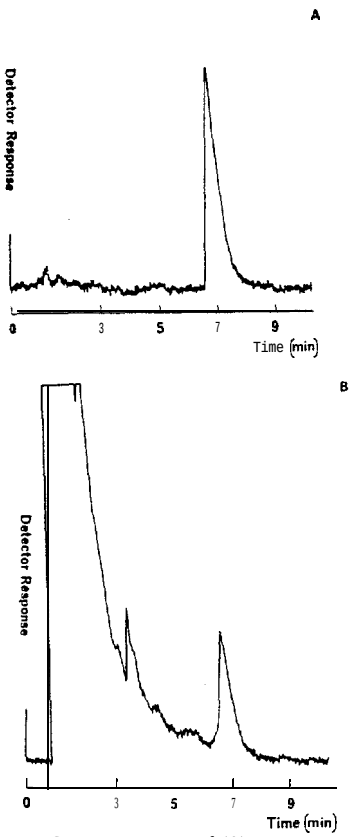

Figure 1. HPLC chromatograms of (A) pure mogroside V and (B) mogroside V as a constituent of an aqueous extract of *Thladiantha grosvenori* whole fruits, purchased in Hong Kong. For operating conditions, see text.

Although we found the isolation of mogroside V from *T.* grosuenori fruits in completely pure form to require considerable attention, it may be seen from Figure 1 that adequate resolution of this compound from other polar constituents was achieved by using the HPLC methodology described here, which involved comparatively little sample cleanup. After controlled experiments to determine the effectiveness of the extraction procedure, the recovery of a standard amount of mogroside V was determined as 82%. The concentration levels of mogroside V in two samples of authenticated *T. grosvenori* fruits available to us were sample 1 (purchased in Hong Kong), whole fruits, 1.29% w/w, peel, 1.26% w/w, endocarp, 1.56% w/w, seeds, 0.24% w/w and sample 2 (purchased in Chicago), whole fruits, 0.80% w/w, peel, 0.81% w/w, endocarp, 1.37% w/w, seeds, 0.51% w/w. Therefore, in both cases, the presence of this compound was observed at the highest concentration levels in the endocarp, when compared with other parts of the fruit. Also, we have been able to confirm the statement of Lee (1975) that the rind (peel) as well as the

pulp of Lo Han Kuo fruits contains a sweet principle.

Takemoto and co-workers (1983a) have reported the isolation of two other sweet triterpene glycoside constituents of *T. grosvenori* fruits in addition to mogroside V, namely, mogrosides IV and VI, which have molecular weights of 1124 and 1448 dalton, respectively. While mogroside VI was found to occur in very small quantities, mogroside IV was obtained in a yield almost as high as mogroside V (Takemoto et al., 1983a). During our work on *T. grosvenori* fruits, we have not observed by analytical TLC or HPLC either mogroside IV or any sweet triterpene glycoside less polar than mogroside V. However, since mogrosides IV and V are clearly separable by reversed-phase HPLC, in which a 25 cm X 4 mm Nucleocil C_{18} column was eluted with 42% ethanol (Takemoto et al., 1983a), it is not expected that significant amounts of mogroside IV in *T. grosvenori* samples will affect the validity of the present HPLC assay for mogroside V.

In other work performed in this laboratory on mogroside V, this compound has been shown to be nonmutagenic and to produce no mortality in acute toxicity experiments on mice at doses up to 2 g/kg body weight and to exhibit an equivalent molar sweetness intensity to the *ent*-kaurene glycoside, stevioside, when tested against a standard sucrose solution by a human taste panel (Kinghorn et al., 1985, unpublished results). These attributes, coupled with the high mogroside V levels in dried 7'. *grosvenori* fruits that are reported here, could serve to stimulate further study as to the suitability of extracts of the fruit of this plant and its constituents as alternative high-intensity sweeteners. It has been suggested already that *T. grosvenori* may be a suitable species for introduction into the United States (Swingle, 1941).

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Changes in the Polypeptide Composition of Peanut (*Arachis hypogaea* L.) Seed during Oil Roasting

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Free amino acids and sugars, released during roasting, are known to be major flavor precursors in roasted peanuts and they give rise to pyrazine compounds via Millard sugar-amine type reaction. In order to identify the protein/polypeptide source of these amino acids, peanut (*Arachis hypogaea L.*) seeds of Virginia, Runner, and Spanish market types were roasted in peanut oil for O-12 min and protein was extracted and examined by gel filtration and gel electrophoresis. Gel filtration studies indicated that roasting caused a decrease in the methionine-rich proteins and aggregation of arachin proteins. Gel electrophoresis studies also showed a decrease in the methionine-rich protein and their polypeptides. In addition, a polypeptide with a molecular weight of 70 000 also gradually decreased during roasting. In contrast. the protein and polypeptide composition of arachin remained relatively unchanged during the 12-min roasting period. It is suggested that the polypeptide/s of methionine-rich protein may be involved in the formation of pyrazine compounds.

The sugar-amine nonenzymatic browning reaction has been shown to be involved in roasted peanut flavor formation (Pickett and Holley, 1952; Newell et al., 1967; Mason et al., 1969). Free amino acids and free sugars which are released during roasting are known to be major flavor precursors in roasted peanuts (Newell et al., 1967) and they give rise to pyrazine compounds via Millard sugar-amine type reaction (Mason et al., 1967; Johnson et al., 1971; Walradt et al.. 1971). Koehler et al. (1969) investigated the pathway for the formation of alkylated

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Stimulation of the Gerbil's Gustatory Receptors by Some Potently Sweet Terpenoids†

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The gerbil was investigated as a model for sweet taste among several highly sweet plant terpenoids and the sweet dihydroisocoumarin phyllodulcin. Although the gerbil'schorda tympani nerve did not respond in electrophysiological experiments to rebaudiosides B and C, steviolbioside, and phyllodulcin, concentration-response curves were obtained for the stimulatory sweeteners hernandulcin, mogroside V, periandrin III, rebaudioside A, and stevioside. These compounds were more effective stimuli in the gerbil than sucrose, in the following order of potency: rebaudioside A = stevioside = periandrin III > hemandulcin > mogroside V > sucrose. In conditioned-taste aversion studies, gerbils trained to avoid these five stimulatory compounds generalized an avoidance to sucrose but not to hydrochloric acid, and except for the perception of a concomitant salty taste, our data show that these substances taste like sucrose to gerbils, as in humans. Support is thus provided *for* the potential involvement of this methodology to guide the purification of natural sweeteners from plant extracts.

There is an increasinginterest in highly sweet nonnutritive and noncariogenic natural sweeteners, and over 50 such substances in more than 15 structural classes are biosynthesized by members of the plant kingdom (Kinghorn and Soejarto, 1986, 1989). Several sweet plant constituents, in either pure or partially purified form, are used commercially as sucrose substitutes in Japan, including the terpenoid glycosides glycyrrhixin, mogroside V. stevioside, andrebaudioside A, thedihydroisocoumsrin phyllodulcin, and the protein thaumatin (Ishikawa et al, 1991; Kinghorn and Compadre, 1991). In addition, semisyntheticcompounds based on plant constituentssuch as neohesperidin dihydrochalcone and perillartine are approved sweeteners in a number of countries (Horowitz and Gentili, 1991; Kinghorn and Compadre, 1991).

In electrophysiological gustatory experiments using the Mongolian gerbil, many different classes of naturally occurring and synthetic sweet compounds have been shown to stimulate the animal's taste nerve, including sweet monosaccharides, disaccharides, and polyols, as well as *more* potently *sweet substances such as* chlorosucrose, L-cyanosuccinanilic acid, dulcin, sodium saccharin, stevioside, and 6-chloro-D-tryptophan (Jakinovich, 1976, 1981; Jakinovich and Goldstein, 1976; Jekinovich and Oakley, 1976). Furthermore, the behavioral conditioned aversion technique has proven to be successful in rodent studies to identify the taste qualities of many compounds, such as alcohols, amino acids, aspartame, sodium saccharin, and sugars (Garcia et al., 1974; Herness and Pfaffmann, 1986; Jakinovich, 1981, 1982; Kasahara et al., 1987; Kiefer and Lawrence, 1988; Myers et al., 1989; Nachman and Cole, 1971; Ninomiya et al., 1984; Nissenbaum and Scafani, 1987; Nowlis et al., 1980; Pritchard and Scott, 1982; Smith and Theodore, 1984; Spector and Grill, 1988; Steward and Krafczek, 1988; Thomesen et al., 1988). Accordingly, using sweet compounds found to stimulate the gerbil's chorda tympani nerve in electrophysiological experiments, the majority of such substances were found to resemble sucrose in behavioral experiments using the Mongolian gerbil. However, some of these sweet substances were not avoided by animals trained to avoid sucrose, so it cannot be assumed that all compounds that are "sweet" to man are 'sweet" to the gerbil (Jakinovich, 1981, 1982a,b).

In an ongoing program to discover novel highly sweet natural products (Kaneda et al., 1992), it has been our practice to subject extracts of sweet-tasting plants to acute toxicity tests in mice and bacterial mutagenicity testing, prior to evaluation for sweetness by human participants. Toinvestigate thepossibilityofcircumventingsuchacostly and rather inconvenient safety procedure, we have found that a combination of gerbil electrophysiological and conditioned taste aversion experiments could be used in a generally reliable fashion to detect the presence or absence of sweet-tasting terpenoid glycosides in extracts of different polarities of three well-known sweet-tasting plants, namely, *Abrus precatorius*, *Stevia rebaudiana*, and *Thladiantha grosvenorii* (Jakinovich et al., 1990). The present study extends our previous effort by investigating the effect on the gerbil's receptors of several of the purified sweet-tasting diterpene constituents of S. *rebaudiana* (rebaudiosides A-C, stevioside, steviolbioside) (Kinghorn and Soejarto, 1986, Tanaka, 1982) and the major triterpene glycoside sweet principle of *T.* grosoenorii (mogroside V) (Takemoto et al.. 1983). In addition, several other pure plant-derived sweeteners have been evaluated in the gerbil model: hernandulcin, a sesquiterpene constituent from *Lippia dulcis* (Compadre et al.. 1985); periandrin III, a triterpene glycoside from *Periandra dulcis* (Hashimoto et al., 1982); and phyllodulcin, a dihydroisocoumarin obtained from the crushed or fermented leaves of *Hy-*

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drangea macrophylla var. Thunbergii (Arakawa and Nakaraki, 1959, Kinghorn and Soejarto, 1986). Since many of these potent natural sweeteners were observed in the present study to stimulate the gerbil's chorda tympani nerve and to taste like sucrose in behavioral experiments., further support has been obtained for the inclusion of experiments on the gerbil in the fractionation of sweet-tasting plants, thereby partially offsetting the need for human volunteer subjects.

MATERIALS AND METHODS

General Procedures. Melting points were determined on a Kofler hot-stage instrument and are uncorrected. Optical rotations, W, IR, ^{1}H NMR, ^{13}C NMR, and low-resolution mass spectrometry were performed as described previously (Kaneda et al., 1992). Analytical TLC was carried out on Merck silica gel G plates, with 250 μm thick layers, which were visualized in short-wave W light and/or after spraying with 60% w/v H_2SO_4 and heating at 110 °C for 10 min.' Where suitable, HPLC analysis was performed to further verify purity (Makapugay et al., 1984, 1985; Compadre et al., 198'7).

Test Compounds. Hernandulcin (1) wee synthesized in the racemic form by directed-aldol condensation from 3-methyl-2-cyclohexen-l-one and 6 methyl-5-hepten-2-one and purified as previously described. This compound wee indistinguishable spectroscopically and chromatographically from its naturally occurring (+)form, (6*S*,1′*S*)-hernandulcin (Compadre et al., 1985, 1987). Mogroside V (2) wee purified from Lo Han Kuo fruits [*T. grosvenorii* (Swingle) C. Jeffrey; recently renamed *Siraitia grosvenorii* (Swingle) C. Jeffrey1 (Kinghorn and Compadre, 1991) end characterized as described previously (Makapugay et al., 1985). The trisodium salt of periandrin III (3) was generously donated by Yamasa Shoyu Co., Ltd. (Chosi, Chiba, Japan) and, after conversion to the parent compound and workup, exhibited physical and spectroscopic data identical to published values for periandrin III (3) (Hashimoto et al., 19821.

The dihydroisocoumarin, phyllodulcin (4), was isolated from the crushed *leaves* of *H. macrophylla* Seringe var. *Thunbergii* (Siebold) Makino, kindly supplied by the late Prof. T. Takemoto. An initial methanol-water (4:1) extract was prepared from the dried milled plant material (7 kg) from which the solvent was removed. On suspension in water and partitioning with ethyl acetate, the ethyl acetate residue (350 g) was purified by gravity column chromatography over silica gel (Merck, Darmstadt, Germany), and phyllodulcin wee eluted with mixtures of petroleum ether-chloroform in proportions of 13:7 and 1:1. Isolated phyllodulcin (4, 90 g, 1.28% w/w) was recrystallized as white needles from chloroform–petroleum ether [mp 118–119 °C, $[\alpha]^{25}_D$ +80.5° (*c* 2.9, $CHCl_3$) [lit mp 119–121 °C; $[\alpha]^{25}_D$ +70.7–80.8° (*c* 1.02, Me_2CO)] (Arakawa and Nakazaki, 195911 end exhibited spectroscopic date closely comparable to published values for this compound (Suzuki et al., 19781. The identity of 4 as phyllodulcin was confirmed by direct comparison (mmp, EI-MS, ^{1}H NMR, co-TLC) to a reference sample kindly supplied by Prof. M. Yamamoto.

Rebaudioside A (5), rebaudioside C (7), and stevioside (9) were isolated and characterized from S. *rebaudiana* (Bertoni) Bertoni leaves, as described previously. Rebaudioside B (6) and steviolbioside (8) were obtained from compounds 5 and 9, respectively, by alkaline hydrolysis. All five of these sweet diterpene glycosides exhibited physical end spectroscopic date consistent with literature values (Makapugay et al., 1984).

Prior to being used in the present study, all compounds were tested for purity by analytical TLC and/or HPLC. The structures of test compounds l-9 are shown in Figure 1.

Experimental Animals. Mongolian gerbils (*Meriones unguiculatus*) were obtained from Tumblebrook Farms, West Brookfield, MA. For electrophysiological experiments, male and female animals, and were less than 1 year old and weighing 50–70 g, were used. For behavioral experiments, male gerbils aged 7-12 weeks were utilized and were 50–60 g in weight.

Methods. *Electrophysiological Methods.* (*a*) *Anesthetic.* Gerbils were injected with ketamine as the primary anesthetic because it produces complete anesthesia in 5-10 min. The ketamine (100 mg/mL) was injected ate dose of 330 mg/kg into the gerbil's thigh muscle. If an animal required further anesthetic during the experiment, sodium pentobarbital (5 mg/mL; 0.15 mL) injected intraperitoneally was employed (Somenerain end Jakinovich, 1990).

(*b*) *Electrophysiology.* Each animal was secured to a head-holder (Oakley and Schaffer, 19X) which immobilized the skull.

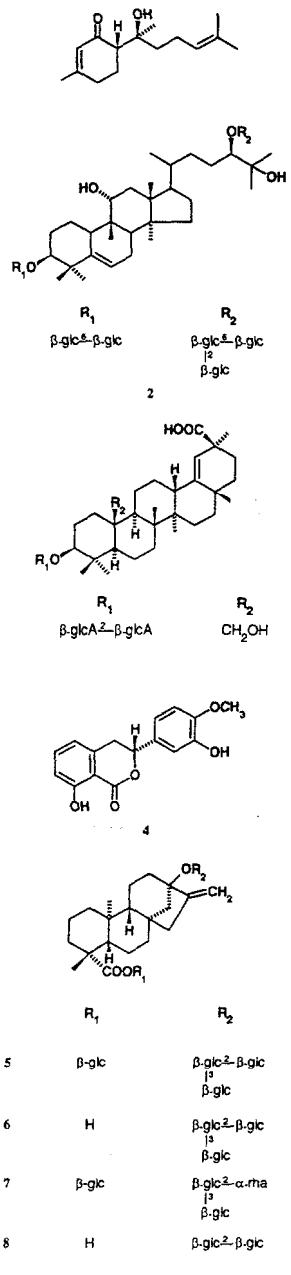

Figure 1. Chemical structures of the highly sweet terpenoids (1–3, 5–9) and the dihydroisocoumarin (4) investigated in this study. Sugar unite: β-glc = β-D-glucopyranosyl; β-glcA = β-D-glucuronopyranosyl; a-rbe = α-L-rhamnopyranosyl.

The method for exposing and recording from the intact chorda tympani nerve has been reported in detail (Somenerain and Jekinovich, 1990).

(c) Stimulation. Chemical stimulation of the tongue was effected by a gravity-flow funnel-tubing system through which deionized water flowed continuously (0.13–0.17 mL/s). Test solutions (2–4 mL) were alternated with water without interruption of the flow. The temperatures of the water and the taste solutions were identical, 25 ± 1 °C. Each compound was tested twice, before and after a standard. Whenever the standard solution elicited responses differing by more than 15%, all interjacent responses were rejected.

(d) *Taste Solutions. All* compounds were dissolved in deionized tap water (>1 megohm). When not used immediately, the sweetener solutions were stored in frozen form or at 2 °C for later use, when they were brought to room temperature.

(e) *Mixtures.* To determine if nonstimulating sweeteners were interacting with the sweetener taste receptor sites, responses to mixtures of sucrose and a nonstimulating sweetener and of rebaudioside A and a nonstimulating sweetener were compared to responses to sucrose end rebaudioside A (Jakinovich, 1981, 1983; Vlahopoulos and Jakinovich, 1988).

Behavioral Methods. (a) General Scope of Study. This behavior study comprised two sets of experiments, with the first to determine how the gerbils perceived the taste of mogroside V (2, 0.001 M) and stevioside (9, 0.002 M). The second set of experiments dealt with the perception of the taste by the gerbil of hernandulcin (1, 0.01 M), periandrin III (3, sodium salt) (0.003 M), and rebaudioside A (5, 0.002 M). The concentrations used were the CR_{50}'s (concentrations that produced half-maximal responses), as determined from the appropriate electrophoretic concentration-response curve, or else the maximum solubility obtained in water.

(b) *Conditioned Taste Aversion.* The following training procedures were used in all experiments:

(1) *Water Intake* Training. One day after arrival, the animals were housed in individual plastic cages with wood chip bedding, rather than in individual wire-bottom cages, to avoid health problems (Jakinovich, 1981.1982; Jakinovich et al., 1990; Myers et al., 1989). Two days later, all test animals were placed on a drinking schedule whereby they received deionized water twice daily (from 09:00 to 10:00 am. and from 3:00 to 4:00 p.m.). Animals were fed Purina Rat Chow (Ralston Purina Co.) *ad libitum.*

(2) *Conditioned Avoidance Training. After6* days of the water training, the animals were randomly divided into three groups of 12 each, with the first group trained to avoid mogroside V (2, 0.001 M), the second group trained to avoid stevioside (9, 0.002 M), and a control group *trained* to avoid water. The mogroside V (0.001 M) and stevioside (0.002 M) solutions were used as conditioning solutions in the following manner: On the Friday of each of the *consecutive* weeks, during the usual morning drinking period, one group of gerbils was offered a drinking bottle containing mogroside V, one group was offered stevioside, and the third group, acting as control, received only water. These solutions were offered for 15 min during the morning watering period. When each animal finished drinking, its drinking tube was again placed in its mouth, leaving a few drops behind, and, immediately after that, the gerbil was injected with LiCl(0.3 M) at 1% of its body weight. Shortly after the injections, the animals showed lethargic appearances which lasted for several hours. This entire avoidance training procedure, as described above, was repeated on the following Friday, and on the Friday of each consecutive week.

(3) *Conditioned Avoidance Testing. On* the Monday of the third week, having allowed 2 days for the animals to recuperate, half of the animals in each group were offered water bottles containing sucrose (0.03 Ml, while the other half received bottles containing NaCl solution (0.01 M). Then, the following morning the animals received these two solutions in reverse order. On Wednesday and Thursday. the above procedure was repeated. Measurements of the amounts of particular fluids consumed were made by weighing the drinking bottles immediately after each animal stopped drinking. During the afternoon drinking period, all animals received only deionized water.

On the Monday of the fourth week, 50 % of the animals in each group were offered water bottles containing HCl (0.01 M), while the other half received battles of quinine hydrochloride solution (0.001 M). Next, on the following morning, the animals received those solutions in reverse order. On Wednesday and Thursday, the above procedure was repeated.

To strengthen and reinforce the aversion, the entire conditioning and testing was repeated. The entire above-indicated behavioral procedure was repeated with solutions of hernandulcin (1, 0.01 M), periandrin III (3, sodium salt, 0.003 M), end rebaudioside A (5, 0.002 M).

(c) *Statistics.* One-way analyses of variance (ANOVA) were applied to the behavioral results. When significant differences were observed, pairwise analyses (*t*-test) were performed between the control and experimental groups (see Results).

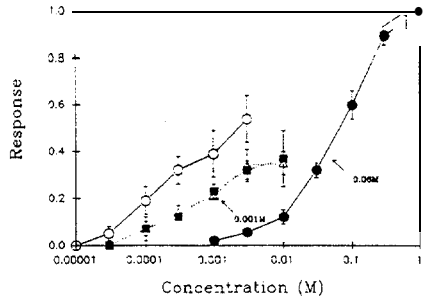

Figure 2. Integrated neural discharge from the gerbil's chords tympani nerve in response to various concentrations (log scale) of purified rebaudioside A (△), Nutrilite stevioside (impure commercial source) (○), purified stevioside (■), *and sucrose* (●). *The* CR_{50}*'s are* shown. Bars indicate ±2SE; *N* for sucrose = 14, N for purified stevioside = 5, and N for rehaudioside A = 8.

RESULTS

No Electrophysiological Responses. The following compounds did not produce responses in the gerbil's chorda tympani nerve at the concentrations used: phyllodulcin (4), rebaudioside B (6), rebaudioside C (7), and steviolbioside (8). Their maximum solubility in water ranged from 1 x 10^{-3} to 5 x 10^{-4} M.

Electrophysiological Responses—Concentration–Response Curves Determined. Hernandulcin (1), mogroside V (2), periandrin III (3, sodium salt), rebaudioside A (5), and stevioside (9) produced responses in the gerbil's chorda tympani nerve. The maximum solubility of the compounds in water was around 0.01 M, which was lower than that of sucrose in all cases. Responses to two compounds, rebaudioside A and stevioside, that produced concentration–response curves with a shape similar to that of sucrose, are shown in Figure 2. These concentration curves exhibited a sigmoidal shape with a maximum response (R_{max}) evident, which is a characteristic of a normal neural sweetener taste response (Jakinovich and Sugarman, 1989). The R_{max} values of rebaudioside A and stevioside were 0.4 (sucrose R_{max}, 1.0). The previously published concentration-response curve of stevioside obtained from a commercial source (Nutrilite Products Inc., Buena Park, CA) is included in Figure 2 for comparison purposes (Jakinovich, 1981). Since R_{max} values were determined, CR_{50} data could be used as one measure of potency (Jakinovich. 1976), and the results obtained were rebaudioside A CR_{50} = *0.001* M, stevioside CR_{50} = 0.001 M, and sucrose CR_{50} = 0.06 M. Another measure of potency, the K_d value (dissociation constant, representing the efficacy of the sweet compounds) (Biedler, 1954), was determined for three sweeteners using the reciprocal plot, as expressed in Figure 3 (sucrose, 0.07 M; stevioside, 0.0013 M; rebaudioside A, 0.0014 M). A third measure of potency, threshold value, was determined directly from Figure 2

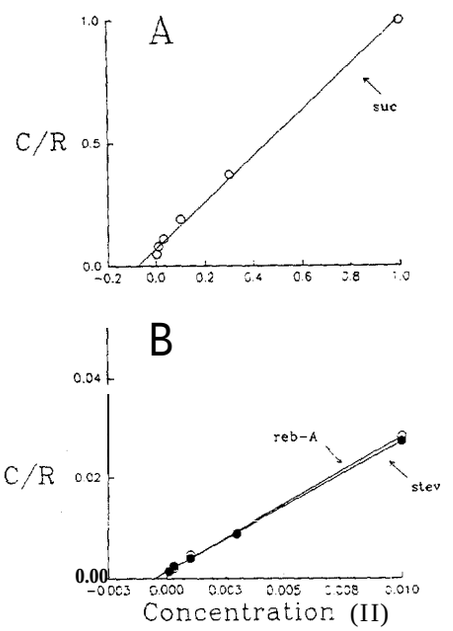

Figure 3. (A) Reciprocal plot of gerbil integrated chords tympani nerve responses to sucrose (suc, ○). (B) Reciprocal plot of gerbil integrated chorda tympani nerve responses to rebaudioside A (reb-A, 0) and stevioside (stev, ●). C = concentration, R = response, slope = $1/R_{max}$, K_d/R_{max} = y intercept, and $-K_d$ = x intercept.

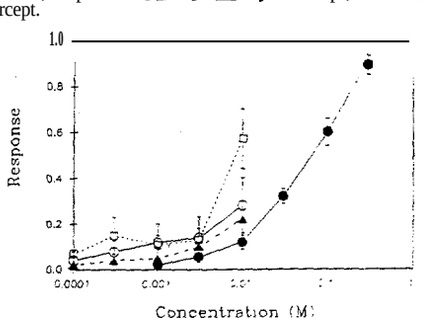

Figure 4. Integrated neural discharge from the gerbil's chorda tympani nerve in response to various concentrations (log scale) of hernandulcin (A), mogroside V (□), periandrin III (sodium salt) (○), and sucrose (●). Bars indicate ±2SE. N for sucrose = 14, N for hernandulcin = 5, N for mogroside V = 6, and N for periandrin III (sodium salt) = 5.

(sucrose, 0.003 M; stevioside, 0.0001 M; rebaudioside A, 0.0001 M).

We were unable to obtain complete sigmoidally shaped (with R_{max}) concentration-response curves for three compounds, hernandulcin (1), mogroside V (2), and periandrin III (3; sodium salt) (Figure 4). However, since the R_{max}'s were not present, the potency of each compound was determined from its K_d in the reciprocal plot (Figure 5) (Beidler, 1954), with the following values obtained: periandrin III, sodium salt, K_d = 0.0006 M; mogroside V, K_d = 0.003 M; hernandulcin, K_d = 0.002 M. Thresholds were determined from Figure 4: periandrin III (sodium salt) threshold, 0.0001 M; mogroside V threshoid. 0.0001 M; and hernandulcin threshold, 0.0003 M.

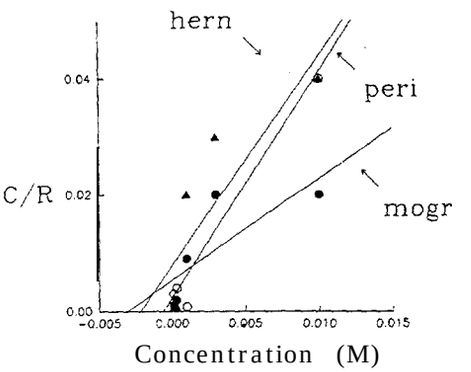

Figure 5. Reciprocal plot of gerbil integrated chorda tympani nerve responses to hernandulcin (hem, Δ), mogroside V (mogro, ●), and periandrin III (sodium salt) (peri, 0).

Mixtures. The taste responses to sucrose or rebaudioside A were not influenced by the presence of a nonstimulating sweetener.

Behavioral Responses. In all cases, gerbils trained to avoid an intense natural sweetener consumed significantly less sucrose (0.03 M) than water (Table I). Concerning saltiness, gerbils trained to avoid hernandulcin, rebaudioside A, and periandrin III (sodium salt) consumed significantly less NaCl (0.01 M) than water. For sourness, intakes of HCl (0.01 M) by all trained gerbils were no differentfromwater. Finally, for bitterness, gerbils trained to avoid mogroside V, hernandulcin, rebaudioside A, and periandrin III (sodium salt) consumed significantly less quinine hydrochloride (0.001 M) than water.

DISCUSSION

In the present gerbil electrophysiological investigation, the CR_{50} in the standard sucrose concentration-response curve waa 0.06 M, which is in general agreement with our past gerbil studies in which the sucrose CR_{50} values ranged from 0.015 to 0.05 M (Somenerain and Jakinovich, 1990). Another consistency was that the sucrose concentration-response curve was sigmoidally shaped and reached an R_{max} (Figure 2). However, it was found in this study that the plant-derived sweeteners phyllodulcin (4), rebaudiosides B and C (6, 7), and steviolbioside (8) did not stimulate the gerbil's chorda tympani nerve in electrophysiological experiments (Table II). These observations are consistent with our previous gerbi! study in which it was discerned that a lack of response in this regard was evident with the following natural and artificial potently sweet compounds: aspartame, beryllium acetate, furan acrylonitrile, 4-(methoxymethyl)-1,4-cyclohexadiene-1-carboxaldehyde syn-oxime, monellin, 5-nitro-2-propoxyaniline, and perillartine (Jakinovich, 1981).

The electrophysiological concentration-response curves of the purified stevioside (9) and rebaudioside A (5) used in this investigation were also sigmoidally shaped and reached an R_{max} of 0.4 (Figure 2). The CR_{50} of both these compounds was 0.001 M. The concentration-response curve of our previously published work using a commercially available stevioside sample is anomalous because it did not reach an R_{max} and was not sigmoidal (Jakinovich, 1981). This distortion is probably due to the presence of unspecified impurities, which are also thought to be responsible for distorted concentration-response curves in the gerbil's chorda tympani nerve responses reported for methyl β-D-galactopyranoside (Jakinovich, 1985) or in human psychophysics studies for D-ribitol (Jakinovich and

Table I. Amounts (Milliliters ± Standard Error) of Test and Control Solutions Consumed by Gerbils Trained To Avoid Highly Sweet Plant-Derived Constituents

conditioning solution	control solution	test solution: 0.03 M sucrose	0.1 M NaCl	0.01 M HCl	0.001 M quinine hydrochloride
mogroside V (0.001 M)		0.66 ± 0.21[a]	3.19 ± 0.27	1.22 ± 0.22	[illegible]
stevioside (0.02 M)		[illegible] ± 0.06[a]	3.32 ± 0.31	1.06 ± 0.17	1.01 ± 0.21
	water–sucrose	207f0.29			
	water–NaCl		2.71f0.18		
	water–HCl			1.63 ± 0.07	
	water–quinine hydrochloride				[illegible] ● 0.12
hernandulcin (0.01 M)		0.95 ± 0.22[a]	1.52 ± 0.35[a]	1.6 ± 0.38	0.76 ± 0.17.
rebaudioside A (0.02 M)		0.21 ± 0.01'	1.05 ± 0.31[a]	1.1 ± 0.16	0.69 ± 0.23[a]
periandrin III (sodium salt) (0.003 M)		1.14 ± 0.27[a]	1.12 ± 0.34[a]	1.6 ± 0.31	0.90 ± 0.19[a]
	water–sucrose	1.81 ± 0.16			
	water–NaCl		2.33 ± 0.33		
	water–HCl			1.85 ± 0.25	
	water–quinine hydrochloride				1.58 ± 0.18

[a] Significant difference, $p > 0.01$; $N = 12$.

Table II. Gerbil Electrophysiological Responses to Stimulatory Plant-Derived Sweeteners[a]

compound	threshold, M	K_d, M	CR_{50}, M	sweetness to humans[c] (× sucrose)	ref
sucrose	0.003	0.07	0.06	1	Beck (1974)
mogroside V	0.0001	0.002	ND[d]	340	Takemoto et al. (1983)
hernandulcin	0.0003	0.003	ND[d]	1,500	Compadre et al. (1985)
periandrin III (sodium salt)	0.0001	0.0006	ND[d]	so	Kinghorn and Soejarto (1986)
rebaudioside A	0.0001	0.0006	0.001	240	Kasai et al. (1981)
stevioside	0.0001	0.0006	0.001	140	Kasai et al. (1981)

[a] Test compounds are ranked according to K_d. [b] The following compounds in the study were nonstimulatory at the doses specified: phyllodulcin (5 x 10^{-4} M); rebaudioside B (1 x 10^{-3} M); rebaudioside C (1 X 10^{-3} M); steviolbioside (1 X 10^{-3} M). [c] Figures are expressed as approximate sweetness intensities on a weight comparison to sucrose. [d] ND, not determined.

Sugarman, 1989). The reduced R_{max}'s observed in the stevioside and rebaudioside A concentration-response curves when compared to that of sucrose suggested that stevioside and rebaudioside A either are partial agonists at the receptor site or else bind at a different receptor site from sucrose (Ariëns et al., 1964). Complete concentration-response curves were not obtained for mogroside V (2), periandrin III (3; wed as the sodium salt), and hernandulcin (1). In spite of this, it was possible to rank the potency of these sweeteners as gustatory stimuli in the gerbil on the basis of their determined K_d values in the following order of decreasing magnitude: rebaudioside A = stevioside = periandrin III (sodium salt) > hernandulcin > mogroside V > sucrose (Table II). A similar ranking list of potency was obtained by considering threshold and CR_{50} values.

The results of the behavioral experiments showed that the gerbil's taste responses to hernandulcin, mogroside V, periandrin III (sodium salt), rebaudioside A, and stevioside resemble their effects in humans. To the human, representatives of these highly sweet natural sweeteners, for which hedonic data have been reported, have been found to taste either sweet (resembling the taste of sucrose) or sweet-bitter (resembling the taste of sucrose and quinine) (Compadre et al., 1985; Schiffman et al., 1979). In the human, all five of these natural sweeteners are sweeter than sucrose, in the following order of potency of sweetness intensity: hemandulcin > mogroside A > rebaudioside A > stevioside > periandrin III > sucrose (Table II) (Compadre et al., 1985; Kasai et al., 1981; Klghom and Soejarto, 1986; Takemoto et al., 1983). When the two ent-kaurene glycoside sweeteners in this group of stimulatory compounds are considered structurally, rebaudioside A possesses one more glucopyranosyl moiety in its C-13-affixed saccharide unit than stevioside, which accordingly confers greater sweetness and more pleasant hedonic attributes for humans. However, the sweetness potency of rebaudioside A is greatly diminished in human subjects either by removal of the glucose attached to C-19 or by substitution of a glucose moiety by rhamnose in the C-13 sugar unit, as in rebaudiosides B and C, respectively (Kinghom and Soejarto, 1986; Tanaka, 1982).

In conclusion, a combination of two well-established gerbil electrophysiological and behavioral assays has been applied to a structurally diverse group of terpenoids that are highly sweet to humans, constituted by the biaabolane sesquiterpene hemandulcin, the cucurbitane triterpene glycoside mogroside V, the oleansne-type triterpene glycoside periendrin III, and the ent-kaurene-type diterpene glycosides rebaudioside A and stevioside. It is significant that the most abundant sweet constituents of *T. grosvenorii* fruits (mogroside V) and S. *rebaudiana* leaves (stevioside and rebaudioside A) have been found to *stimulate* the gerbil's taste receptors in this study, since we have earlier shown that extracts containing these compounds also gave positive data (Jakinovich et al., 1999). Although these gerbil assays do not respond to all classes ofcompoundsperceivedassweet byhumans, they do seem to have validity for evaluating sweet-tasting terpenoids of plant origin.

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